EFFICACY OF AUTOLOGOUS PLATELET RICH FIBRIN (PRF) DRESSING OVER MOIST SALINE/POVIDONE IODINE DRESSING IN DIABETIC FOOT ULCERS AND THE INFLUENCE OF TLR 4 RECEPTORS IN THEIR HEALING

A RANDOMISED CONTROLLED STUDY

Dissertation submitted to

THE TAMILNADU DR. M.G.R. MEDICAL UNIVERSITY, CHENNAI

In partial fulfillment of the degree of

M.S. GENERAL SURGERY

Branch- I

PSG INSTITUTE OF MEDICAL SCIENCES AND RESEARCH, COIMBATORE

DEPARTMENT OF GENERAL SURGERY

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CERTIFICATE

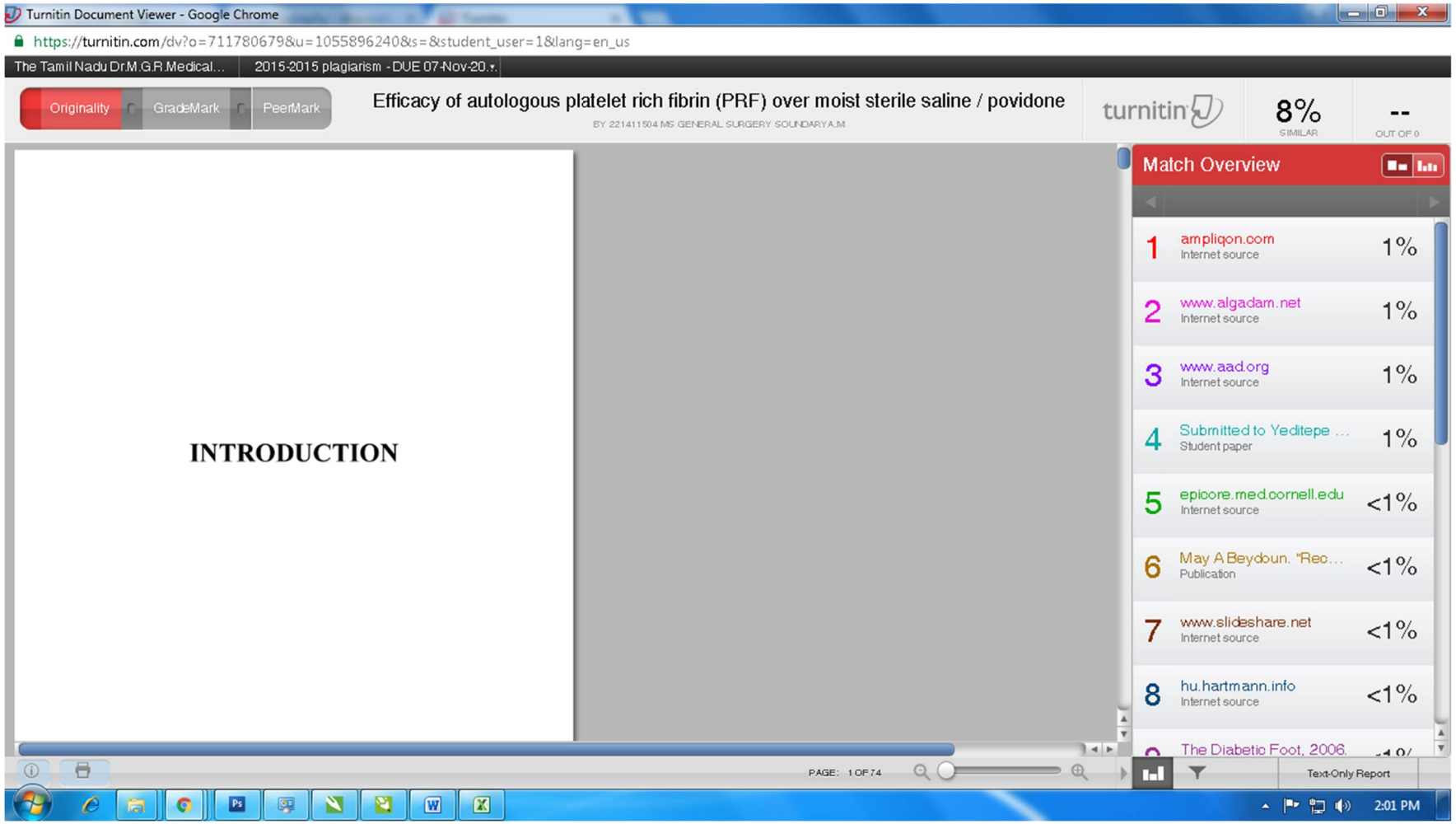
This is to certify that **DR.SOUNDARYA.M** postgraduate student (2014-2017) in the department of General Surgery, PSG INSTITUTE OF MEDICAL SCIENCES AND RESEARCH, Coimbatore has done this dissertation titled **"EFFICACY OF AUTOLOGOUS PLATELET RICH FIBRIN (PRF) DRESSING OVER MOIST SALINE/POVIDONE IODINE DRESSING IN DIABETIC FOOT ULCERS AND THE INFLUENCE OF TLR 4 RECEPTORS IN THEIR HEALING"** under the direct guidance and supervision of guide **Prof .DR.VIMAL KUMAR GOVINDAN** and co-guide **DR. THIAGARAJAN** in partial fulfilment of the regulations laid down by the **Tamilnadu Dr.M.G.R. Medical university**, Chennai, for M.S., Branch – I General Surgery degree examination.

Prof.DR.VIMAL KUMAR GOVINDAN M.S,FRCS
Chief Unit II
Dept.of.General Surgery
PSGIMS&

Associate Prof. DR. THIAGARAJAN
Dept of Molecular Medicine &
Therapeutics
PSGIMS&R

Prof. DR. S. PREM KUMAR MS
Professor & Head
Dept. of General Surgery
PSG IMS&R

Prof . DR. S. RAMALINGAM.M.D
DEAN
PSG IMS&R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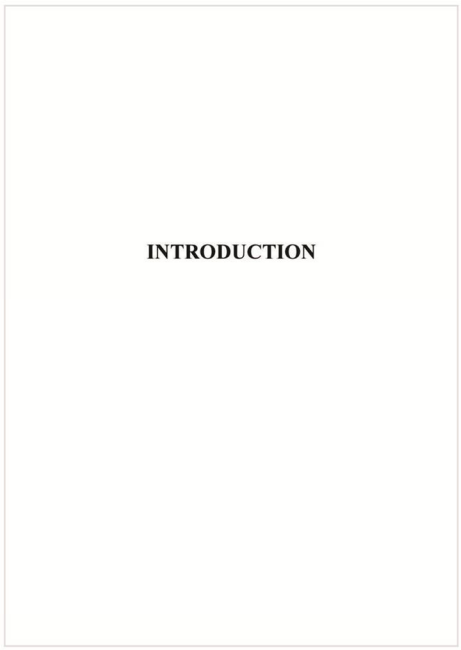

INTRODUCTION

PSG Institute of Medical Sciences & Research
Institutional Human Ethics Committee

Recognized by The Strategic Initiative for Developing Capacity in Ethical Review (SIDCER)
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Phone : 91 422 - 2598822, 2570170, Fax : 91 422 - 2594400, Email : ihec@psgimsr.ac.in

To
Dr M Soundarya
Postgraduate
Department of General Surgery
PSG IMS & R
Coimbatore

Ref: Project No.14/454

Date: March 27, 2015

Dear Dr Soundarya,

Institutional Human Ethics Committee, PSG IMS&R reviewed and discussed your application dated 19.12.2014 to conduct the research study entitled *"Efficacy of autologous platelet rich fibrin (PRF) over moist sterile saline / povidone iodine dressing in diabetic foot ulcers and the influence of TLR 4 receptors in their healing - a randomized controlled study"* during the IHEC meeting held on 19.01.2015.

The following documents were reviewed and approved:

1. Project Submission form
2. Study protocol
3. Informed consent form
4. Data collection tool
5. Current CVs of Principal investigator, Co-investigator
6. Budget

The following members of the Institutional Human Ethics Committee (IHEC) were present at the meeting held on 19.01.2015 at IHEC Secretariat, PSG IMS & R between 10.00 am and 11.00 am:

Sl. No.	Name of the Member of IHEC	Qualification	Area of Expertise	Gender	Affiliation to the Institution Yes/No	Present at the meeting Yes/No
1	Dr. P. Sathyan (Chairperson, IHEC)	DO, DNB	Clinician (Ophthalmology)	Male	No	Yes
2	Dr. S. Bhuvaneshwari (Member-Secretary, IHEC)	MD	Clinical Pharmacology	Female	Yes	Yes
3	Dr. S.Shanthakumari	MD	Pathology, Ethicist	Female	Yes	Yes
4	Dr. D. Vijaya	M Sc, Ph D	Basic Medical Sciences (Biochemistry)	Female	Yes	Yes

The study is approved in its presented form. The decision was arrived at through consensus. Neither PI nor any of proposed study team members were present during the decision making of the IHEC. The IHEC functions in accordance with the ICH-GCP/ICMR/Schedule Y guidelines. The approval is valid until one year from the date of sanction. You may make a written request for renewal / extension of the validity, along with the submission of status report as decided by the IHEC.

PSG Institute of Medical Sciences & Research
Institutional Human Ethics Committee

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POST BOX NO. 1674, PEELAMEDU, COIMBATORE 641 004, TAMIL NADU, INDIA
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Following points must be noted:

1. IHEC should be informed of the date of initiation of the study
2. Status report of the study should be submitted to the IHEC every 12 months
3. PI and other investigators should co-operate fully with IHEC, who will monitor the trial from time to time
4. At the time of PI's retirement/intention to leave the institute, study responsibility should be transferred to a colleague after obtaining clearance from HOD, Status report, including accounts details should be submitted to IHEC and extramural sponsors
5. In case of any new information or any SAE, which could affect any study, must be informed to IHEC and sponsors. The PI should report SAEs occurred for IHEC approved studies within 7 days of the occurrence of the SAE. If the SAE is 'Death', the IHEC Secretariat will receive the SAE reporting form within 24 hours of the occurrence
6. In the event of any protocol amendments, IHEC must be informed and the amendments should be highlighted in clear terms as follows:
   a. The exact alteration/amendment should be specified and indicated where the amendment occurred in the original project. (Page no. Clause no. etc.)
   b. Alteration in the budgetary status should be clearly indicated and the revised budget form should be submitted
   c. If the amendments require a change in the consent form, the copy of revised Consent Form should be submitted to Ethics Committee for approval
   d. If the amendment demands a re-look at the toxicity or side effects to patients, the same should be documented
   e. If there are any amendments in the trial design, these must be incorporated in the protocol, and other study documents. These revised documents should be submitted for approval of the IHEC and only then can they be implemented
   f. Any deviation-Violation/waiver in the protocol must be informed to the IHEC within the stipulated period for review
7. Final report along with summary of findings and presentations/publications if any on closure of the study should be submitted to IHEC

Kindly note this approval is subject to ratification in the forthcoming full board review meeting of the IHEC.

Thanking You,

Yours Sincerely,

SECRETARY
PSG IMS&R
COIMBATORE-641004

Dr S Bhuvaneshwari
Member - Secretary
Institutional Human Ethics Committee

DECLARATION

I, **Dr.SOUNDARYA.M.**, solemnly declare that this dissertation **"EFFICACY OF AUTOLOGOUS PLATELET RICH FIBRIN (PRF) DRESSING OVER MOIST SALINE/POVIDONE IODINE DRESSING IN DIABETIC FOOT ULCERS AND THE INFLUENCE OF TLR 4 RECEPTORS IN THEIR HEALING"** is a bonafide record of work done by me in the Department of General Surgery, PSG Institute of Medical Sciences and Research, Coimbatore, under the guidance of **Prof. DR. VIMAL KUMAR GOVINDAN, M.S, FRCS.**

This dissertation is submitted to the Tamilnadu Dr.M.G.R.Medical University, Chennai in partial fulfillment of the University regulations for the award of MS Degree (General Surgery) Branch-I, Examination to be held in April 2017.

Place: Coimbatore

Date:

Dr.SOUNDARYA.M

ACKNOWLEDGEMENT

I wish to thank PSG HOSPITALS for having permitted me to conduct this study in this hospital.

I am ever grateful to all the faculty members of Department of General Surgery and Department of Molecular Medicine & Therapeutics, PSG IMS&R for their generous help, kind guidance, valuable advice, expert supervision & encouragement throughout my career & for the preparation for this dissertation.

Last but not the least, I express my gratitude to all the patients for their cooperation for being a part of my study, my colleagues and parents for their support and blessings, without whom nothing would have been possible in this world.

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INTRODUCTION

Diabetic foot ulcer is a devastating complication of diabetes mellitus. This condition is more common in old patients. The risk in a diabetic patient's lifetime having this complication of diabetic foot ulcer is estimated to be about 15%. Majority of diabetic foot ulcers may heal whereas a smaller percentage will remain active and finally lead to amputation of the limb.

With increasing duration of the ulcer and the increasing age of the patient, the risk of amputation also rises. Early prevention plays a vital role in betterment of the quality of life of the patient and also reduces the financial toll.

Diabetic foot ulcer is one of the most demanding problems and it is recommended that a multi disciplinary team work is essential for management. Wound dressings constitute a major part of the management of diabetic foot ulceration. An ideal dressing will have to reduce the symptoms, give adequate protection of the wound and promote good healing.

There are many types of dressings available for the treatment of foot ulcers. There is no particular dressing that fulfils all the requirements of the patient. Platelet rich fibrin prepared from patients own blood is under

extensive research and is used in the fields of orthopaedics and dentistry to promote wound healing. Platelet rich fibrin transfers growth factors to the wound surface. The use of an autologous preparation reduces the risk of allergic reactions and promotes delivery of many growth factors when compared to conventional preparations. There are many studies on the use of platelet rich fibrin as a dressing alternative, but only very few studies compare the efficacy of PRF with other dressing materials.

The normal wound healing process involves a series of stages namely acute inflammation, proliferation and remodeling. The innate immune system plays a major role in wound healing cascade and is usually mediated by toll like receptors. The down regulation of these receptors influence the normal healing pathway. The many chemokines and cytokines released have anti microbial effect. Toll Like Receptors were first discovered in 1985 by Christiane Nusslein- Volhard. The first discovered member of the TLR family was the toll like receptor 4 which is responsible for the release of a great range of cytokines.

TLR 4 helps in immune-stimulation, inflammation, angiogenesis , tissue repair and regeneration and is recently under study for its major role in wound healing. Patients with down regulation of these receptors take longer time to heal.

This also helps in expression of Vascular Endothelial Growth Factor. Therefore when there is differential expression of TLR 4 there is impaired wound healing.

This study will compare the efficacy of PRF dressing with moist saline/povidone iodine dressing in diabetic patients having foot ulcers. Only a limited number of studies have been done to compare the efficacy of PRF dressing with other conventional methods. The molecular part of this study was done to determine the influence of TLR polymorphism in wound healing.

ABSTRACT

AIM:

To compare the efficacy of autologous platelet rich fibrin (PRF) over moist sterile saline / povidone iodine dressing in diabetic foot ulcers and the influence of TLR 4 receptors in their healing – a randomized controlled study.

OBJECTIVES:

(I) To compare the mean reduction in ulcer area at the end of 4 weeks of dressings.

(II) To study the influence of expression of TLR 4 polymorphism in wound healing.

METHODOLOGY

60 diabetic patients with foot ulcers from the department of general surgery, general medicine, endocrinology, and cardiology were prospectively studied. Detailed clinical history, evaluation of ulcer and presence of wound infection were assessed for all the patients.

Patients were randomized into two groups of 30 patients each. While one group received PRF dressings, the other received saline/povidone iodine

dressings. The wound healing was then compared in the two groups. Also the influence of TLR 4 receptor polymorphisms were studied in two different genes TLR 911 and TLR 914, in both saline and PRF treated groups.

The efficacy of platelet rich fibrin dressing over moist saline/povidone iodine dressings was assessed by comparing the percentage reduction at the end of four weeks, using chi square test and ANOVA test. The influence of TLR 4 receptor polymorphism in wound healing was also studied in 13 patients who received PRF dressings and 13 patients who received moist saline/povidone iodine dressings and analyzed using chi square test and ANOVA test.

RESULTS

It was found that there was a better reduction in the area of the ulcer at the end of four weeks in patients who received platelet rich fibrin dressings than in moist saline/povidone iodine dressings. The molecular study performed showed better healing with expression of AG genotype in Toll like receptors but it was statistically insignificant. The limitation of this aspect of the study was that it was a small sample size.

CONCLUSION:

The use of platelet rich fibrin dressing showed better reduction in ulcer area at the end of four weeks when compared with moist saline/povidone iodine dressings. AG genotype expression showed better healing than AA or GG genotype expressions in TLR 911 and TLR 914. However the molecular study did not show a statistically significant reduction.

REVIEW OF LITERATURE

Diabetic foot has been one of the most feared chronic complications of diabetes mellitus. Incapacitating consequences like limb amputation has made such fears justified. Morbidity associated with diabetic foot problems, in quite a few patients, reach a point of no return.

Thus, early diagnosis and institution of intensive management become essential. Early identification of the foot at risk of developing major problems, is vital, in order to bring about prompt preventive measures.

LOUDON in 1805, had quoted that, “leg ulcers are generally looked upon as an inferior branch of practice, an unpleasant and inglorious task where much labour must be bestowed and little honour gained”

HISTORY:

During the ancient times and the middle ages, diabetes was a rare disease. The misconception of diabetic foot as gangrene lasted for several years and this resulted in aggressive surgical management with major amputation of the leg above the knee.

ANTIQUITY: Aretaeus, The Cappadocian (130-200), disciple of Hippocrates was the first to describe symptoms of diabetes and the first to use the term “diabetes” from the Greek word that refers a siphon.

THE RENAISSANCE: French military surgeon Ambroise Pare (1510-1590) used ligatures to control bleeding after amputation and bandages to cover wounds.

18TH CENTURY: By this time, diabetes mellitus was no longer a rare disease. Matthew Dobson (1735-1784) showed the presence of sugar in the urine and blood of patients with diabetes. William Cullen (1710- 1790), established the difference in between diabetes mellitus which had the colour, smell and flavor of honey in urine and diabetes insipidus which had limped but the urine was not sweet.

19TH CENTURY: Marchal de Calvi (1852) recognized the association between diabetes and gangrene, which hitherto was considered to be one and the same. He also drew attention to the causal relationship between diabetes and disorders of the nervous system.

Frederick Treves (1884) described the clinical history of a neuropathic foot ulcer. He recommended bed rest and sharp debridement followed by the application of salicylic acid, glycerine and carbolic acid cream following which he applied a thick felt pad.

Only in 1893, the difference between a gangrene caused by any infection in a part with normal blood supply and the gangrene caused by vascular insufficiency was found.

20^{TH} CENTURY: Maurice J. Lewi (1857-1957) recognized the medical establishments' indifference towards the "minor foot ills" of mankind. In 1913 he founded "The school of chiropody" now known as "The New York college of podiatric medicine"

The therapeutic era in the history of diabetes dawned in the 1920s with Frederick Banting's discovery of the insulin.

In 1934, Elliot P Joslin in his paper entitled; "The Menace of Diabetic Gangrene" noted that deaths from gangrene foot and leg had risen significantly. "That gangrene is heaven-sent but is earth born" (1)

21^{ST} CENTURY: In the first decade of the 21^{st} century diabetes has emerged from what has been a rare disease during the age of antiquity and the Middle Ages to a global epidemic.

On November 14, 2005, World diabetes day, the International Diabetes Federation launched a yearlong campaign to highlight the need for urgent action to bring about improvement in diabetic care.

Of the many serious complications of diabetes mellitus, history has shown that foot complications take the greatest toll.

EPIDEMIOLOGY:

Nearly 30 percentage of the patients with diabetes mellitus have a high risk for developing foot ulcers. It has been estimated that in each year nearly 3 to 7 % of patients with diabetes will develop an ulcer in the foot.

Foot ulcers occur in approximately 15% of people with diabetes which accounts for 25% of all hospital admissions. The hospital stay is 60% longer than the stay for other causes and the risk of amputation is very high in diabetics than in others [(2)].

Diabetic foot ulcers constitute for nearly more than half of the amputations performed due to a non traumatic cause. Diabetes is responsible for high mortalities, need for amputation more than once and also the need for amputation in the other limb[(2)].

India has 30 million diabetics at present and the numbers are growing. It is predicted that in the year 2025 India will have 57 million people suffering from diabetes mellitus[(2)].

Incidence in India[(2)]:

Foot Ulcer: 1-4%

Toe amputation: 2.6%

Below knee amputation: 1.6%

Prevalence of diabetic foot in India: 5.3 - 10.5%

Socio – economic impact:

Overall, the costs generated by diabetes, and the consequences of the disease, are about three times as high in when compared with patients who are not affected by diabetes. The complications involving the foot will occupy a great amount.[3]

If the wound heals primarily then the cost factor plays a minor role whereas if it progresses to the need of amputation the cost factor also increases. It has been estimated that for a wound to completely heal it may require about 3 months. Of which a small proportion of ulcer will be present for more than a year[4]

The patient also suffers depression and he/she becomes dependant.

PATHOPHYSIOLOGY OF DIABETIC FOOT LESIONS

Ischemia, neuropathy, infection and sustained hyperglycaemia are the principal pathogenic factors.

1. <u>Role of Vasculopathy</u>[5]: Atherosclerosis will be seen earlier in diabetic patients than in age matched non diabetic patient taken as controls.

Diabetic patients have two different type of of arterial changes:

- Large vessel (macroangiopathy)
- Small vessel (microangiopathy).

There are qualitative differences in mucopolysaccharides, calcium and cholesterol compared with non - diabetics.

The macrovascular lesion is "Garden - Variety" atherosclerosis. The disease is much more extensive and more commonly associated with medial calcific sclerosis in diabetics than in non – diabetics.

Diabetic microangiopathy involves arteries smaller than 115 micrometer in diameter. The severity and extent of the small vessel lesion distinguish diabetics from non diabetics.

DIABETIC PERIPHERAL VASCULAR DISEASE HAS A PREDILECTION FOR TIBIO- PERONEAL VESSELS.

2. Diabetic Neuropathy[6]:

Neuropathy plays a significant role in the development of diabetic ulcers. Peripheral neuropathy may well be related to the quality of glycaemic control.

The neuropathic diabetic foot is at greater risk, as there is no protective sensation. Minor trauma is unnoticed until there is significant ulceration, infection or bone injury. Also, because of intrinsic foot muscle atrophy and

secondary foot deformities, there is an alteration in the weight distribution and biomechanics of foot function that leads to pressure points, callus formation and skin breakdown.

3. Other Risk factors[7]:

Other pathogenic factors include hyperlipidemia, hypertension, smoking, and the secondary consequences of hyperglycaemia, obesity, genetic factors and hypercoagulability.

Diabetic foot complications depend on both the duration and severity of hyperglycaemia over years.

Foot deformities such as pes planus, pes cavus, bunionette, claw toe, over riding toe deformity, mallet toe can occur.

The above include risk factors that are identified clinically. Many studies done recently have showed that intense mechanisms, that take place at molecular levels hinder wound healing.

TLR 4 IN DIABETIC WOUND HEALING:

Toll-Like receptor 4 (TLR4) plays a vital role in the immunity, repair of injured tissue and regeneration of damaged tissue. The single nucleotide polymorphisms-SNP, in TLR 4 namely, rs4986790, rs4986791, rs11536858 (merged into rs10759931), rs1927911, and rs1927914 have been found to be

greatly linked with diabetic foot ulcers with the risk of diabetic patients developing diabetic foot ulcers. This association is being studied greatly. These polymorphisms will also the natural wound healing capacity in diabetic patients.

In any patient the normal wound healing will evolve through an inflammatory stage then a proliferative stage and finally to a remodeling stage. These steps are essential for god wound strength and wound approximation. In case of any disturbances in these stages, the wound will not heal well and may evolve into chronic non healing wounds.

Diabetes is found to affect the healing of wound in three ways[8]. It will bring about a reduction in growth factors and the cytokines that help in wound healing.

Diabetes plays a role in bringing about a reduction in all the growth factors. It will increase the pro inflammatory cytokines which will cause extension of the first stage of wound healing, namely, the inflammatory stage. It also increases the enzymes that help in destruction of matrix and this will result in down regulation of new matrix synthesis..

To get good wound healing the stages of inflammation has to be properly organized and should not rapidly progress to either the proliferatory or the remodeling stage. Also the most important factor is whether the wound is

affected by any infectious organism which will decide how the wound will evolve [9]. A long standing inflammatory phase or an infected wound may eventually progress to a non healing chronic wound.

Drosophila, the fly, was found to be having similar toll like receptors as in mammals. Through, production of anti microbial peptides and other cytokines and chemokines synthesis, these receptors are found to mediate the innate immunity.

In the TLR family TLR4 has been under study in an extensive manner, which is essential for the immune reactions. In wound healing process, TLR 4 plays a vital role and in case of any disruption in TLR4 mediated pathways the wound healing process will be affected greatly. The differential expression of TLR 4 in diabetic patients with ulcer has shown to influence wound healing in studies done recently.

In recent studies two single nucleotide polymorphisms which lead to change of the amino acids in the domain of TLR 4 receptors have been found. The two polymorphisms are Asp299Gly (rs4986790) and Thr399Ile (rs4986791). These two influence the TLR 4 mediated functions in many ways. rs11536858, rs1927911 and rs1927914 are another three single nucleotide polymorphisms found to be in association with inflammation.

CLINICAL PATTERN OF DIABETIC FOOT LESIONS

The various lesions seen in diabetic foot lesions are infections, ulcers, gangrene and joint lesions.

1. Infections

Diabetic foot infections can be mild where only the superficial layers namely skin and the sub cutaneous layers are involved. Moderate infection will comprise of deeper involvement of the bones and muscles. Severe infection includes necrotizing fasciitis and any rapidly spreading gangrene with high risk for amputation. These infections can be caused by an array of organisms – bacterial or fungal.

BONE INFECTIONS – OSTEOMYELITIS:

Patients with non healing ulcers should be evaluated for the presence of osteomyelitis. In an ulcer of larger size, that is, when it is larger than two cm or if it has a depth of more than 3 mm it has a high chance of having acquired osteomyelitis, when this is supported by neutrophilic leucocytosis or high inflammatory markers.

When a chronic recurrent perforating ulcer is neglected for a long time, the bacteria invade the base of the lesion, then the fascial plane, and later the periosteum, leading to osteomyelitis[(10)].

2. Ulcers

Often associated with infection, diabetic ulcers are classified as

a. Neuropathic ulcers

b. Ischemic ulcers

c. Neuro ischemic ulcers

Neuropathic ulcers

- In regions with increased plantar pressures - metatarsal heads, plantar aspect of the great toe, heel and over bony prominences.
- In most of the patients diabetic neuropathy is present.

These are painless.

- Callous formation is found in the edges of the ulcers. The base is always healthy and granulates well
- When foot is examined there will be peripheral neuropathy – reduced sensation. There will also be loss of sensation to light touch, pain, temperature and vibration sense. There will also be loss of Achilles tendon reflexes. The sensory loss differs from one patient to another.
- On palpation the foot will be normal or sometime warmth may be present.
- Palpable peripheral arterial pulses will be present.
- The ankle brachial pressure index is normal.

Ischemic ulcers:

- This type constitutes a majority of diabetic foot ulcers. These are very painful.
- The edges are red, base may be necrotic – blackish. The patient gives a history of intermittent claudication.
- Inspection and palpation of the limb shows signs of ischemia namely pallor, shiny skin, cool to touch, loss of hair over skin, absence of peripheral arterial pulses.
- The ankle – brachial index is less than 0.9

3. Gangrene

Gangrene implies death with putrefaction of macroscopic portions of the tissue.

Gangrene of Toes:

This is by far the most commonly noted type of lesion. Ooften starts from an unnoticed minor injury. It is usually of the wet type when there is infection but dry type of gangrene is also seen, especially when there is associated vascular disease.

Gangrenous Patches:

These occur in the pressure areas of the foot, most commonly over the heel, the 1st metatarsal medially, and the base of the 5th metatarsal laterally. Small areas of gangrene are also seen in non pressure areas due to atheromatous debris. They are also seen in the interdigital clefts, which are often missed during a routine examination.

Diabetic Gangrene:

This is a term specifically given to a gangrene of a fully vascularised foot. It is usually rapid on onset, and painless with large areas of necrosis. There may be associated systemic illnesses. Signs of deep infection are present but the striking feature is that the ankle pulses will be well felt.

4. Joint lesions:

A very serious complication of diabetes is Charcot osteoarthropathy. This is also called as diabetic neuropathic osteo arthropathy (DNOAP)[(11)]. The cause for this complication to develop is secondary to peripheral autonomic and somatic neuropathy. The blood supply is however adequate. The prevalence of this join lesion is estimated to be 60 years and mostly occurs in patients with a long history of diabetes. There is no gender predilection.

CLASSIFICATION:

1. Meggitt – Wagner classification [12].

0	Fully epithelialized ulcer.
1	Superficial ulcer involving only upto the dermal layer.
2	Beyond the subcutaneous layer. Exposure of tendon or bone without the presence of osteomyelitic changes. Without abscess formation.
3	Ulcers involving deeper planes with presence of osteomyelitis or with abscess formation.
4	Gangrene when only localized.
5	Extensive gangrene.

2. The University of Texas classification[13]:

Grade	0	1	2	3
A	Fully epithelialized ulcer.	Superficial without the involvement of bone or the tendon.	Wound beyond the tendon.	Wound extending beyond the bone or extending into the joint.
B	Infection +	Infection+	Infection+	Infection+

C	Ischemia+	Ischemia+	Ischemia+	Ischemia+
D	Infection + & ischemia +	Infection + & ischemia +	Infection + & ischemia +	Infection + & ischemia +

3. Edmonds & Foster classification:

In this the ankle brachial pressure index is measured and also the clinical tests are done. Depending on these the foot ulcers are divided into neuropathic ulcers or neuroischemic ulcers.

4. Broadsky

A – there is no ischemia

B – presence of ischemia without gangrene

C – partial gangrene

D – extensive gangrene

5. Macfarlane and Jeffcoate[(14)].

According this system, ulcers are classified on the basis of

- Size of the ulcer – measuring the area and depth of the ulcer
- Sepsis
- Arteriopathy
- Denervation

RISK FACTORS FOR FOOT COMPLICATIONS IN DIABETICS:

The risk factors have been classified after categorization [16].

Risk category

0. The patient has preserved sensations but might have foot deformity.

1. Protective sensation is lost.

2. Protective sensation loss is high. History of presence of any callous ulcers or pressure ulcers.

3. Severe foot deformity or toe deformity with restricted joint mobility

MANAGEMENT OF DIABETIC FOOT LESIONS:

In 1999, the American Diabetes Association recognised several basic principles of diabetic wound healing.

1. Off - loading
2. Debridement
3. Use of appropriate dressings
4. Management of infection either conservatively or surgically.
5. Vascular reconstruction and / or amputation or reconstructive foot surgery when necessary.

1. Off loading or pressure relief devices [15]

Prevention of diabetic foot ulcer is essential with pressure relief footwear. The biomechanics as a result of neuropathy will produce As has already been mentioned, biomechanical changes are a frequent consequence of diabetic neuropathy, resulting in an changed pressure points on the patients sole. Pressure relief hence plays an important role in prevention ulcer formation and also aids in proper healing of an already intact ulcer.

The best way for pressure relief is given by Total contact cast. The principle behind this is the cast will let the redistribution of the weight of the patient in such a way that proper healing of wound is allowed [16,17].

2. Debridement

Aggressive debridement remains the mainstay management of diabetic foot ulcers. The principle being to remove the devitalized/necrosed tissue and to give intact vascularisation and a clean environment for the ulcer to heal well

ypes of debridement are as follows:-

1. Sharp surgical method.
2. Mechanical method – to use wet -to-dry dressings.
3. Enzymatic method – collagenase ,trypsin when the sharp dissection cannot be done.

4. Autolytic method; digests the tissue .
5. Biomechanical method (Biosurgery):

   Treatment with sterile maggots [18] (Larval therapy).

   Leeches are also used in amputation. They form hirudin which has an anti inflammatory and anti thrombotic effect.

After debridement and infection control, the raw area is allowed to heal by (i) Granulation, (ii) Applying Split skin graft or Local random flaps or Pedicled muscle flaps.

3. Dressings:

There a wide range of dressing materials available at present. The advantages and disadvantages of these dressings are given in the table below.

Type of dressing	Advantages	Disadvantages
Traditional dressings (gauze and absorbent cellulose)	Cheap and widely available. Appropriate for gangrenous lesions	Fixes to the wound and may produce bleeding while changing.
Semi-permeable films	Form bacterial barrier. Durable. Require changing only once every 4-5 days. Cheap	Some patients are allergic to the adhesive in the dressing

Foams	Appropriate for ulcers with high production of exudates.	Effect difficult to quantify. Not as effective and rapid as surgical debridement.
Hydrocolloids	Safe and selective, using the body's own defense mechanisms. Good for necrotic lesions, with light to moderate exudates. No slip. Cost – effective	Their occlusive tendency makes wound from being assessed everday. Wound must be monitored closely for signs of infection. May promote anaerobic growth and mask a secondary infection
Alginates	Useful as absorbents of exudates. Better for infected ulcers.	Cannot be used in neuro-ischemic ulcers, which produce minimal exudates. This has the disadvantage of a dry wound. The removal of dressing becomes difficult.
Enzymatic dressings	Ideal for ulcers with large areas. Casuses faster healing and promote autolysis. The incidence of maceration is low.	Costly. Has to be done over the necrotic patches. He/she may have irritation.

PLATELET RICH FIBRIN DRESSING

Role of platelets in wound healing:

After any injury the vital reactions that lead to immediate coagulation are mainly platelet mediated. At the injury site, platelets release many effective inflammatory substances which help in wound healing in many steps of a wound healing process [19]. Growth factors are found to be released when platelets are activated on application over the wound [20]. Platelets' role in the healing of wounds is of great importance and used in various fields of medicine. Activated platelets release growth factors which help in repair of tissue by the process of angiogenesis, collagen production which is an extracellular matrix component, by formation of granulation tissue and re epithelialisation[21].

EVOLUTION OF PLATELET CONCENTRATE:

Many blood components, that help wound healing naturally, have been applied to wounded tissues to promote wound healing faster. Platelet concentrate has become a biological surgical additive with the use of fibrin adhesives [22]. In 1970, fibrin glue was first found. Initially it was done from plasma obtained from donors, but in view of the lesser fibrinogen concentration in plasma, the quality of same was poor. Later the adhesive was obtained from patients own blood autologously.

Platelet rich plasma (PRP), an autolougous preparation of fibrin glue, is produced by steps which aggregate platelets. It is used in several fields to promote better wound healing. Growth factors are present abundantly in PRp as well. The growth factors help in wound healing.

It is an undemanding method to make the platelet concentrate and enhance blot clots naturally. 94% RBCs, 5% platelets, 1% WBCs constitute the natural blood clot. Whereas PRP contains 95% platelets [23]. Thus PRP derived from patients own blood supplements a rich source of growth factors to the region of an injury site or an ulcer that needs natural healing.

This is overcome by the Platelet rich fibrin preparations. PRF is knwn as a second generation platelet concentrate.

PLATELET RICH FIBRIN:

When a wound heals there are high metabolic requirements of the inflammatory reactions that take place. This necessitates to carefully select the appropriate approach that helps in healing of wound in a favourable environment. Platelet Rich Fibrin (PRF) is one of those biological approaches that help in wound healing.

PRF preparations yields a platelet concentrate that aggregates on to a fibrin membrane that favours wound healing. It has abundant growth factors and the use of same has been under extensive study. Application of PRF has

also been reported to be an effective method to initiate tissue regeneration and response. 60 biologically active substances are found in platelets that are helpful in repair mechanisms including cell proliferation, intracellular matrix deposition, chemotaxis, immune modulation, antimicrobial response, angiogenesis and remodelling[24].

The platelet rich fibrin methodology also offers an automated system for convenient preparation and use of autologous platelet rich fibrin which is prepared from patient's own blood in the management of wound. With this concept activated platelets in PRF is used widely in the management of diabetic foot ulcers.

Various studies support the view, that the use of PRF promotes three important steps in wound healing namely angiogenesis, immune response and epithelial proliferation, and its use to protect open wounds and also promote wound healing. Use of PRF is gaining a lot of importance in case of non healing ulcers.

Preparation of PRF:

PRF preparation required a table centrifuge under sterile precautions. 10 ml of patient's own whole blood was drawn into vacutainer tubes without any anticoagulant and centrifuged at 3000 rpm for about ten minutes. In the middle of the tube there was the fibrin clot in between the RBCs at the

bottom and acellular plasma at the top. 10 ml of whole blood yielded about 2.5 ml of clot. This clot was removed from the tube under aseptic precautions with the help of a sterile forceps and the attached RBCs were scraped off and discarded. Fibrin clot was be used for dressing immediately.

The most important factor in this is the time taken for the collection of patient's blood sample, transfer of same to the vacutainer and the centrifuge machine. Without any anticoagulant the sample collected begins to coagulate as soon as the blood comes in contact with the vacutainer. Also the formed fibrin clot has to be obtained from the tube and placed at the site of use within minutes of its formation, for its best action.

PRF VS PRP:

For the following reasons, PRF is better than PRP [25,26].

- Is simple and easy to prepare
- Cost effective
- Bovine thrombin is required to convert fibrinogen to fibrin, while preparing PRP
- The conversion of fibrinogen to fibrin, in PRF, happens slowly with the naturally available thrombin that is present in the patient's own blood sample collected, thus rendering a physiological environment

that is favourable for wound healing. A better cell proliferation and migration takes place that is led by the naturally formed fibrin matrix [27].

- PRF supports immune system
- Due to slow polymerization there is intrinsic entrapment of platelet cytokines in the fibrin mesh. Hence proves the fact that platelet rich fibrin preparations are not as good as the other preparations. It can release platelet cytokines during remodelling phase of healing process [28,29].

The slower release of growth factors from PRF than from PRP and the better healing with the use of plateltet rich fibrin was showed by Dohan *et al*[30]. Studies have showed that PRP tends to emit growth factors and also has less capacity to provoke the regeneration of bone [31]. The platelet rich fibrin's role as a support for the bone protein was studied by Kawamura and Urist. [31] In another study Bensaid*et al* [32] it was found out that the cells are able to move from them fibrin matrix. Bovine thrombin used for PRP preparation has toxic effects on cells.

MATERIALS AND METHODS

This study was conducted to compare the efficacy of autologous PRF with moist saline & povidone iodine dressing in diabetic patients with foot ulcers, by looking at the mean reduction of ulcer area at the end of 4 weeks.

This study was also conducted to compare the polymorphism of TLR 4 receptors in diabetic and non diabetic foot ulcers.

Inclusion Criteria:

Diabetic patients with foot ulcers of lower extremity, having an ulcer area of 1cm x 1cm to 5cm x 5 cm.

Exclusion Criteria:

1. Ulcers of other etiology e.g. ischaemic ulcer/venous ulcer/ulcer with underlying vasculitis.
2. Patients with osteomyelitis affecting the area of the ulcer.
3. Ulcers with exposure of tendons or bones.
4. Platelet count < 1,50,000/ mm3
5. Age of the patient < 18 years
6. Pregnancy and lactation
7. Non-consenting patients

The study was conducted on patients with diabetic foot ulcers who were treated either as in patients or out patients in the departments of General Surgery, General Medicine, Cardiology, Nephrology in PSG Institute of Medical Science and Research Centre. The period of study was between February 2015 and February 2016. It is an open labelled prospective randomized control trial.

A total number of 60 diabetic foot ulcer cases participated in the study. All patients with diabetic foot ulcer of lower extremities, and satisfying the inclusion & exclusion criteria participated in the study.

Written informed consents were obtained from the patients. Detailed clinical history of the patient and other relevant data were collected.

For each of the patients, the following details were entered: age, sex, diabetic status, co-morbid conditions, onset and duration of ulcer and ulcer specifications, haemoglobin , platelet levels, wound culture sensitivity reports ulcer area measurements at the end of each week upto 4 weeks. Each patient was followed up till the end of four weeks.

This study has two parts 1) Clinical and 2) Molecular

The clinical part included two groups of patients.

Group 1 (study group) patients with diabetic foot ulcer receiving Platelet Rich Fibrin (PRF) dressing.

Group 2 (control group) patients with diabetic foot ulcer received conventional saline dressing.

Sample size in each group:

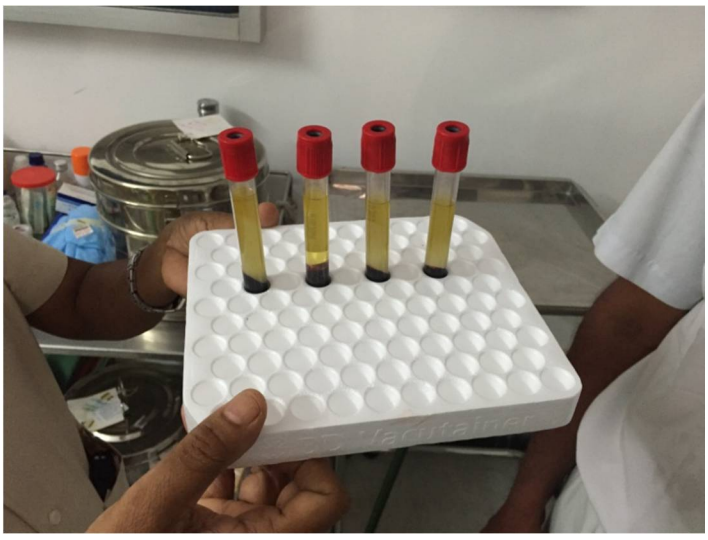
Group 1: 30

Group 2: 30

Preparation of platelet-rich fibrin (PRF):

PRF can be prepared in a simple method and it can be prepared at the time of application. About 10 to 15 ml of patient's blood is collected in sterile vacutainers without any anticoagulant. Then these tubes are kept in a centrifugal machine at the speed of 3000 rpm (revolutions per minute) for about 10 minutes. Two separate layers are formed following centrifugation, the lower part with red blood cells and the upper part with plasma and the fibrin clot. The clot is separated which forms the platelet rich fibrin. The process that happens here is that the upper part of the vacutainer contains fibrinogen that aggregates with thrombin eventually leading to the formation of fibrin. This fibrin clot is formed in the middle part of the tube between plasma on top and RBCs at bottom. Platelets are trapped in the fibrin matrix.

THE CENTRIFUGE MACHINE CONTAINING BLOOD SAMPLES



FOLLOWING CENTIFUGATION SAMPLE SEPARATES TO FORM UPPER FIBRIN CLOT AND LOWER PART OF RBCs

SEPARATED PRF FOR APPLICATION OVER ULCER

Dressing procedure:

Group 1 received PRF clot dressing. It was applied over the wound surface in a thin layer and covered with a sterile saline gauze (primary dressing) followed by cotton pad and roller bandage (secondary dressing). The dressing was left in place for 1 week. After 1 week, all PRF remnants were removed with water and sterile gauze. Following this, the next PRF treatment was instituted. A total of four PRF treatments at weekly intervals were given for a total duration of 4 weeks.

Group 2 received only sterile saline soaked gauze dressing with povidone iodine, which was changed daily.

Baseline measures after randomization:

i) Ulcer margins (length & breadth) in mm: by measuring greatest length and the greatest width.

ii) Area of wound derived with measured length and breadth.

iii) Digital photography taken at beginning and end of treatment.

iv) Outcome measures at the end of four weeks.

Wound swabs were obtained prior to first dressing and patient was started on culture sensitive antibiotics.

For analyzing the results the ulcer area measurements at the end of each week was calculated. The data was collected and entered in the excel spread sheet. The data was analyzed using STATA.

The test variables were compared using chi-square test for two sided independent samples to compare means across dichotomous variables. The one way ANOVA test was done for comparison of means across multilevel variables. Simple calculations like percentages, proportions and mean values were derived. A type I error of 0.05 was considered in all analysis.

The impact of PRF dressings in the healing of diabetic foot ulcers was assessed by analyzing the mean reduction of ulcer area and outcome of the patient, using the above said statistical tools. The statistically significance ($p < 0.1$) was taken into consideration to prove a better outcome in PRF dressings.

MOLECULAR STUDY:

Also the influence of TLR 4 receptor polymorphisms were studied in two different genes TLR 911 and TLR 914. This part of the study also included two groups.

Group 1: TLR 4 polymorphisms in patients with diabetic foot ulcers who received PRF dressing.

Group 2: TLR 4 polymorphisms in patients with diabetic foot ulcers who received saline dressing.

Sample size in each group:

Group 1: 13

Group 2: 13

Storage of blood:

Treated whole blood samples with EDTA, ACD or heparin can be used. It can be frozen or fresh. In case of need of a short term storage, blood is collected in tubes containing EDTA as an anticoagulant, and stored at 2-8 C. It is recommended to store blood sample less than 3 days as DNA degradation may occur.

In case of need for a long term storage, blood is collected in tubes containing a standard anticoagulant (preferably EDTA if high molecular weight DNA is required) and stored at -80 C.

Blood collection & treatment:

For every 1 ml of whole blood sample, 0.1 ml of anticoagulant (0.5M EDTA pH 8.0, or ACD,0.48% citric acid, 1.32% sodium citrate, 1.47% glucose) is added.

Procedures for extraction genomic DNA from blood:

1. Sample preparation
   - If >100 µl of blood is used , 2 volumes of Buffer TBP is added. It is then mixed thoroughly and the tube is let to stand for 1 minute until red cells lyse completely. Then it is centrifuged at 4,000 xg (8,000 rpm) for 1 minute. The supernatant is discarded carefully. The precipitate is washed with 500 µl TE buffer 2 times. Again it is centrifuged at 4,000 xg (8,000rpm) for1 minute during each wash. The final precipitate should appear white and step 2 is carried out.
   - Typical yield is 1-3 µg from 100ul blood sample.
2. 20ul of proteinase K is added and mixed well. 200ul of buffer CL is added, vortexed gently and incubated at 56 C for 10 minutes.
   - The solution should appear clear after complete lysis. If solution still appears cloudy, incubation time is extended until lysis is complete and solution is clear.
   - If RNA-free genomic DNA is required 20 µl RNase A(10mg/ml, not provided with kit) is added, mixed by vortexing, and incubated for 5 minutes at room temperature before continuing step 4.
   - If final reaction volume is more than 500 µl proteinase K usage is increased and/or incubation time extended.
3. 200 µl of 100% ethanol is added to the mixture and mixed thoroughly.

4. Entire component is transferred and applied onto EZ 10 column that is in a 2 ml collection tube. This is allowed to stand for 1 to 2 minutes at room temperature. For 2 minutes at 8000 x g (10000 rpm) this is now allowed to spin.
5. 500 µl of CW1 solution is then added and centrifuged at 8000xg (10000rpm) for 1 minute.
6. 500 µl of CW2 solution is added and centrifuged at 8000 x g (10000 rpm) for 1 minute.
7. The flow through is discarded.
8. The column is then placed in a clean 1.5 ml Eppendorf tube, 30 to 50 µl of CE buffer is added in the centre part of membrane in the column. For 2 to 3 minutes it is the incubated at RT.
9. The column is then centrifuged at 8000x g (10000rpm) for 1 minute to elute DNA from it.
10. The DNA quantity is measured by UV absorption at A_{260} (1.0 OD unit is equivalent of 50 µg). By 0.7% agarose gel the genomic DNA quality is assessed.

Polymerase chain reaction (PCR)

Procedure:

1. Taq 2X Master mix and primers are thawed. All components are placed on ice.
2. The table shows the reaction for a volume of 50 µl. in a final volume of 20 µl, 10 µl of the Taq 2X master mix is used.

Table: Reaction components (reaction mix and template DNA)

Component	Volume/reaction	Final concentration
Taq 2x master mix	25 µl	1X
25mM $MgCl_2$	0 µl (0-7)	1.5mM(0.5-5)
Primer A(10 micM)	1 µl (0.5-5)	0.2µM(0.1-1.0)
Primer B(10 micM)	1 µl (0.5-5)	0.2µM(0.1-1.0)
PCR-grade H_2O	X µl	-
Template DNA	X µl	Genomic DNA:50ng(10-500) Plasmid DNA:0.5ng(0.1-1) Bacterial DNA:5ng(1-10)
Total volume	50 µl	-

3. It is then mixed and dispensed in appropriate volumes into each reaction tubes. The reaction mix s then gently mixed by pipetting it up and down a few times.
4. Template DNA is added to the individual tubes containing the reaction mix.
5. The thermal cycler is programmed according to the manufacturer's instructions.
6. The tubes are placed in the thermal cycler and the reaction is started.
7. The PCR products are run on 2% agarose gels to confirm the presence of bands.

Restriction fragment length polymorphism (RFLP)

The PCR products are subjected to RFLP after conformation on agarose gel. The PCR products are digested with restriction enzymes and run on 15% polyacrylamide gel electrophoresis to observe the restriction digestion pattern and conclude the genotyping results.

TLR4 POLYMORPHISMS

1. rs1927911

FP: CCTGCATGCTCTGCACATG

RP: ACCATGGGAATCCATGCAC

Amplicon length: 245 bp

RFLP:Styl

Types of alleles	Number of fragments
Homozygous GG allele	175+2bp+68bp
Heterozygous GA allele	243 bp+175 bp+68 bp+2bp
Homozygous AA allele	243 bp+2bp

2. rs1927914

FP:ACGTCTAGTCTAGAGCATCA

RP:ATTGGAAGTGCTTGGAGGAT

Amplicon length: 270bp

RFLP: Sphl/Bbul

Types of alleles	Number of fragments
Homozygous GG allele	218bp+52bp
Heterozygous GA allele	270bp+218bp+52bp
Homozygous AA allele	270

METHODOLOGY

AIM	• To compare the efficacy of autologous PRF with moist saline&povidone iodine dressing in diabetic patients with foot ulcers and to study the effect of TLR4 polymorphism in wound healing of foot ulcers in diabetic patients. • To compare the mean reduction in ulcer area at end of 4 weeks. • To ascertain the influence of TLR 4 polymorphism in wound healing.
STUDY DESIGN	An open labelled prospective randomized control trial.
STUDY POPULATION	In patients or out patients from departments of General Surgery, General Medicine, Cardiology, Endocrinology, Nephrology over a period (February 2015-February 2016) were included in the study.

SAMPLE SIZE	60
INCLUSION CRITERIA	Diabetic patients with foot ulcers of lower extremity, having an ulcer area of 1cm x 1cm to 5cm x 5 cm.
EXCLUSION CRITERIA	1. Ulcers of other aetiology e.g. ischemic ulcer/venous ulcer/ulcer with underlying vasculitis. 2. Patients with osteomyelitis affecting the area of the ulcer. 3. Ulcers with exposure of tendons or bones. 4. Platelet count < 1,50,000/ mm3 5. Non-consenting patients
DURATION OF STUDY	1 year
STUDY PERIOD	February 2015 to February 2016

STUDY PROFORMA

Efficacy of autologous platelet rich fibrin over moist sterile saline dressing in diabetic foot ulcers and the influence of TLR 4 receptors in their healing.

1) IDENTIFICATION NO:

2) AGE:

3) SEX:

4) IP NO/OP NO:

5) DATE OF ADMISSION:

6) DATE OF DISCHARGE:

7) COMORBID CONDITIONS:

8) DIABETIC STATUS:

DURATION:

MEDICATION:

COMPLICATION:

9) ULCER DETAIL

A. MODE OF ONSET:

- TRAUMATIC
- SPONTANEOUS
- PRESSURE
- OTHERS

B. DURATION:

10). WOUND OBSERVATION:

- SITE
- SIZE
- SHAPE
- EDGE
- MARGIN
- FLOOR
- BASE
- DISCHARGE
- SURROUNDINGS

11.) INVESTIGATIONS:

- HAEMOGLOBIN
- PLATELET COUNT:
- WOUND CULTURE SENSITIVITY:

12). WOUND AREA MEASUREMENTON WEEK 1 IN CM^2 DATE:

TYPE OF DRESSNG

1. SALINE DRESSING / POVIDONE IODINE DRESSING ( )

2. PRF DRESSING ( )

13). WOUND AREA MEASUREMENTON WEEK 2 IN CM^2 DATE:

TYPE OF DRESSNG

1. SALINE DRESSING / POVIDONE IODINE DRESSING ( )

2. 2. PRF DRESSING ( )

14). WOUND AREA MEASUREMENTON WEEK 3 IN CM^2 DATE:

TYPE OF DRESSNG

1. SALINE DRESSING / POVIDONE IODINE DRESSING ( )

2. PRF DRESSING ( )

15). WOUND AREA MEASUREMENTON WEEK 4 IN CM^2 DATE:

TYPE OF DRESSNG

1. SALINE DRESSING / POVIDONE IODINE DRESSING ( )

2. PRF DRESSING ( )

16). TLR 4 RECEPTOR POLYMOPHISM IN PATIENTS WITH DIABETIC FOOT ULCER RECEIVING PRF DRESSING:

17). TLR 4 RECEPTOR POLYMOPHISM IN PATIENTS WITH DIABETIC FOOT ULCER RECEIVING SALINE DRESSING:

RESULTS

60 diabetic patients both in- patients as well as out-patients, from the departments of general surgery, general medicine, nephrology, endocrinology, and cardiology were included in this study. Consent was obtained.

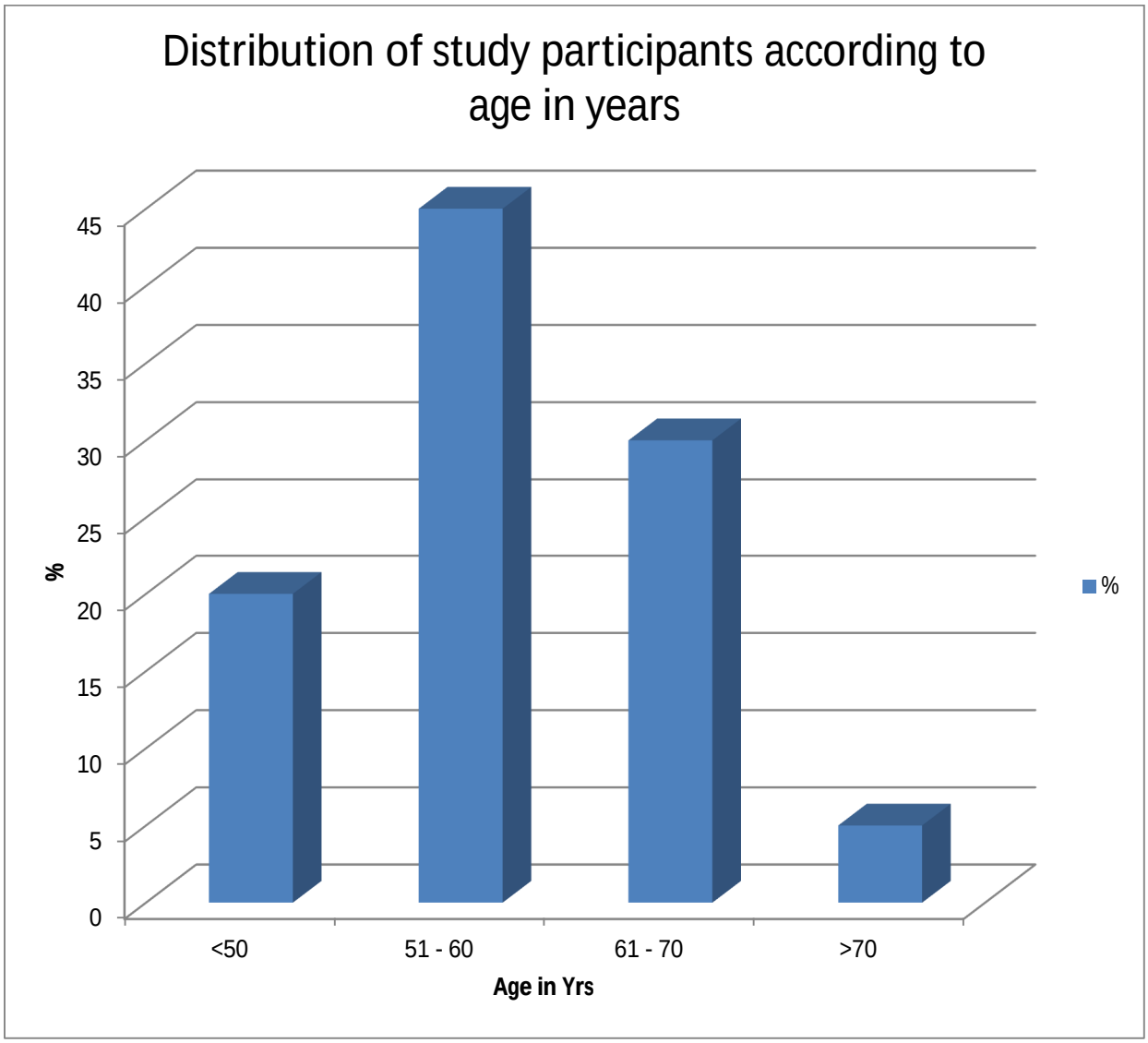
DESCRIPTIVE STATISTICS: AGE

Table 1:

AGE	N	%
<50	12	20
51 – 60	27	45
61 – 70	18	30
>70	3	5
TOTAL	60	

AGE	SALINE	PRF
<50	12	20
<50	4	4
51 – 60	13	13
61 – 70	12	10
>70	1	3

Nearly three quarters of the enrolled patients were in the 6th and 7th decades of life. 20% of the patients were in the younger age group.



GENDER DISTRIBUTION

Table 2:

GENDER	N	%
MEN	41	68.3
WOMEN	19	31.7
TOTAL	60	

GENDER	SALINE	PRF
MEN	21	20
WOMEN	9	10

A significant male preponderance was noted.

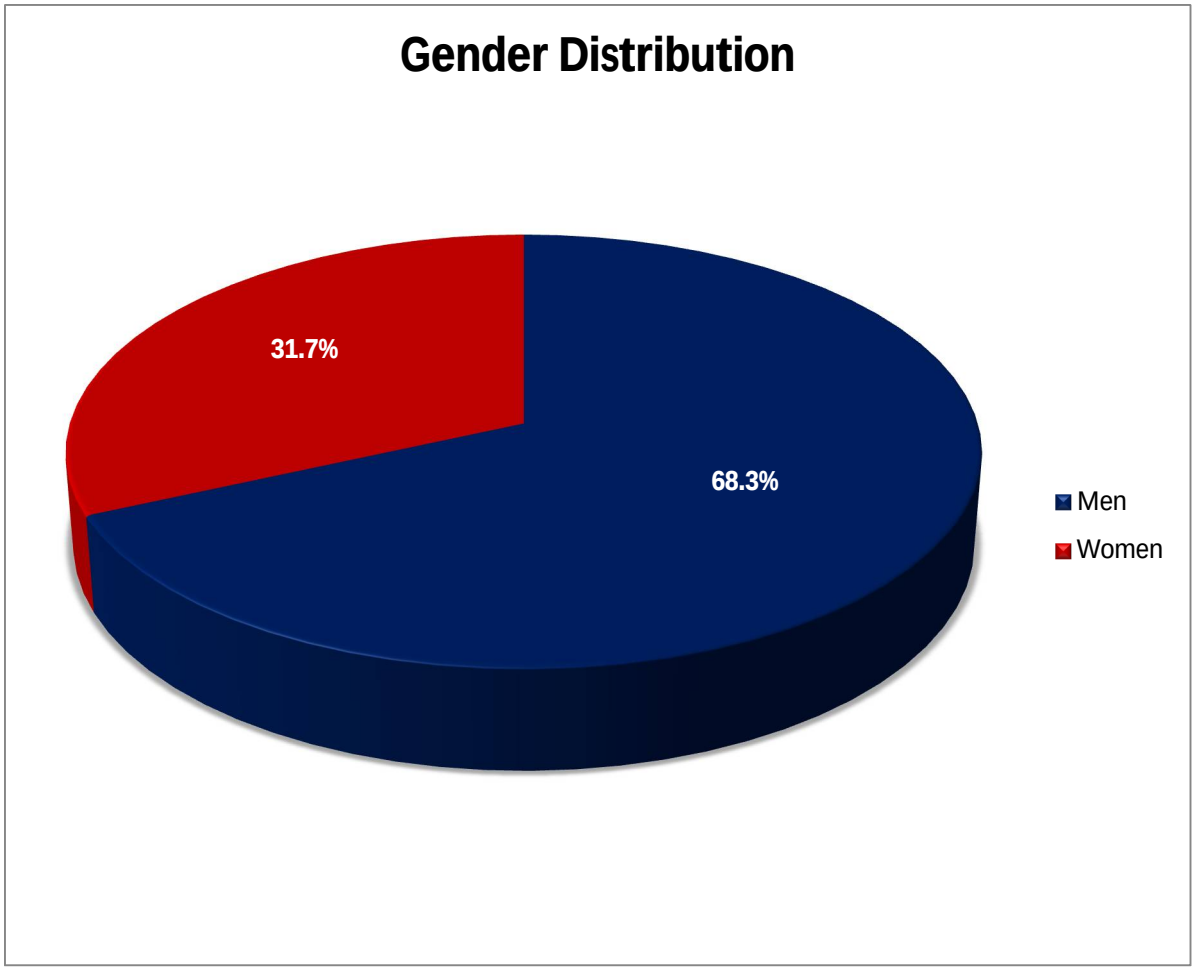

Gender Distribution
31.7%
68.3%
Men
Women

TABLE 3:

CO-MORBID CONDITIONS

COMORBID	N	%
YES	45	75
NO	15	25
TOTAL	60	

COMORBIDS	SALINE	PRF
YES	21	24
NO	9	6

As expected with diabetic patients, only 1 in 4 patients did not have any co-morbid conditions.

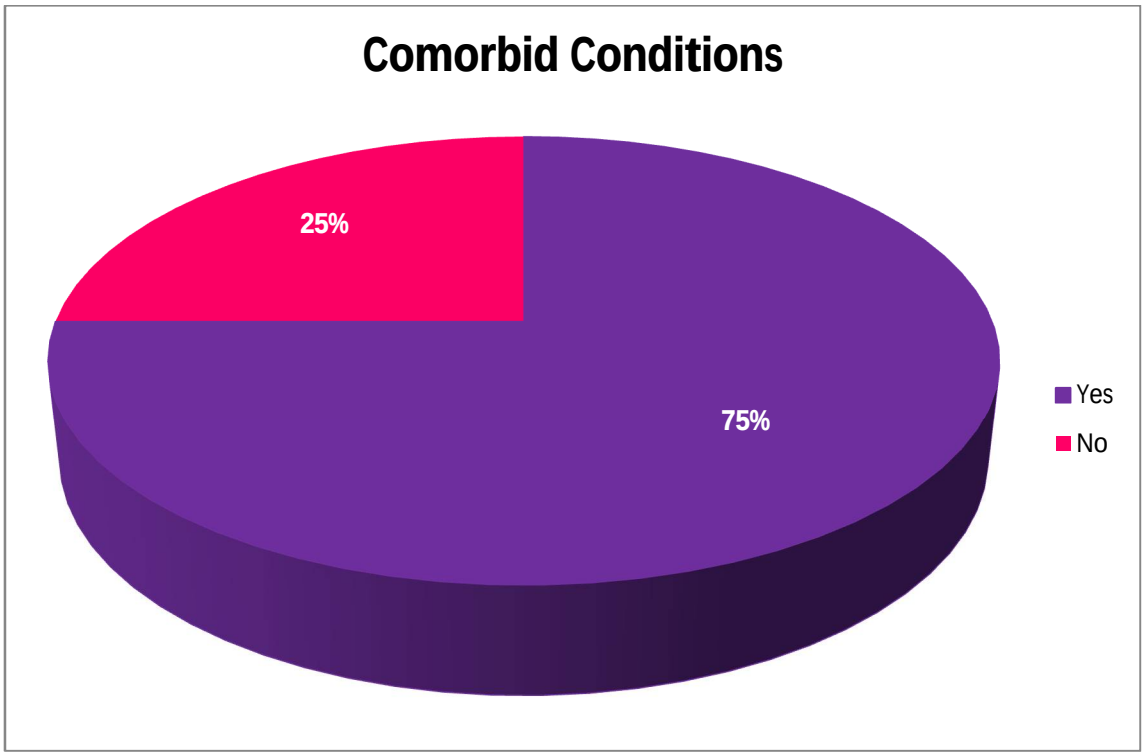

Comorbid Conditions
25%
75%
Yes
No

TABLE 4:

ONSET OF ULCER

ULCER ONSET	N	%
SPONTANEOUS	24	40
POST TRAUMA	36	60
TOTAL	60	

ULCER ONSET	SALINE	PRF
SPONTANEOUS	8	16
POST TRAUMA	22	14

60% of the ulcers were post traumatic whereas 40% had a spontaneous onset.

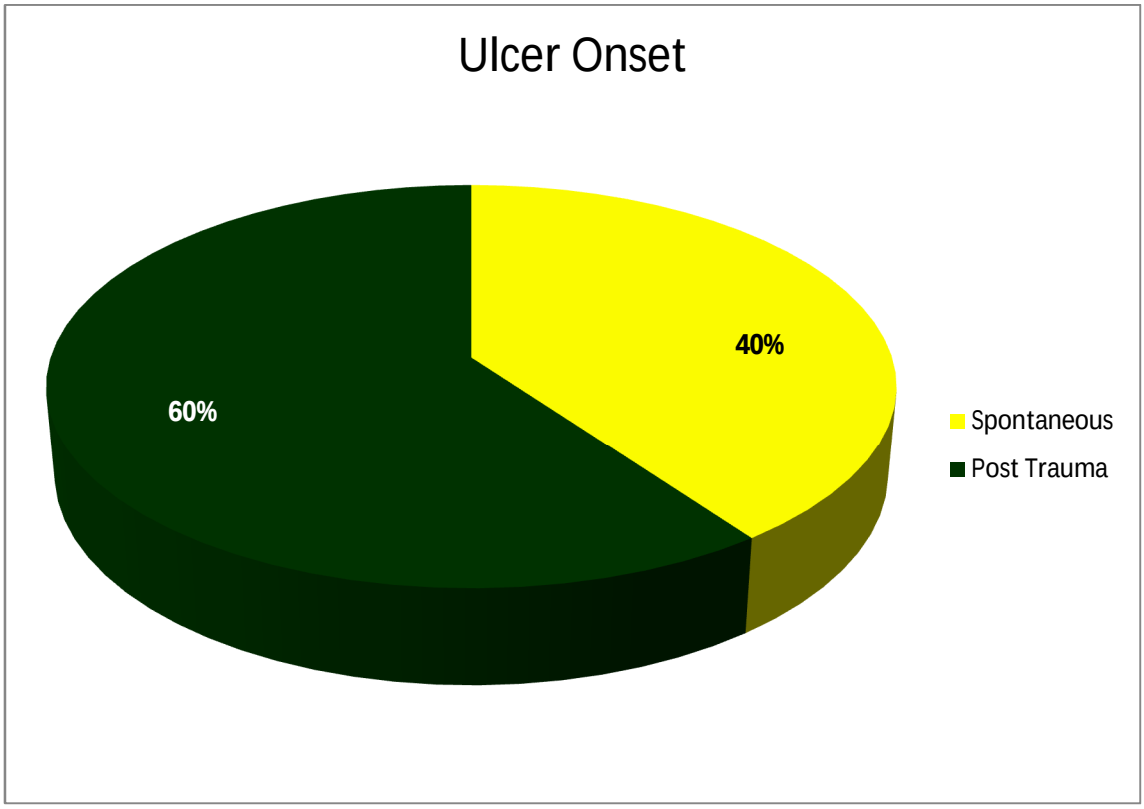

Ulcer Onset
40%
60%
Spontaneous
Post Trauma

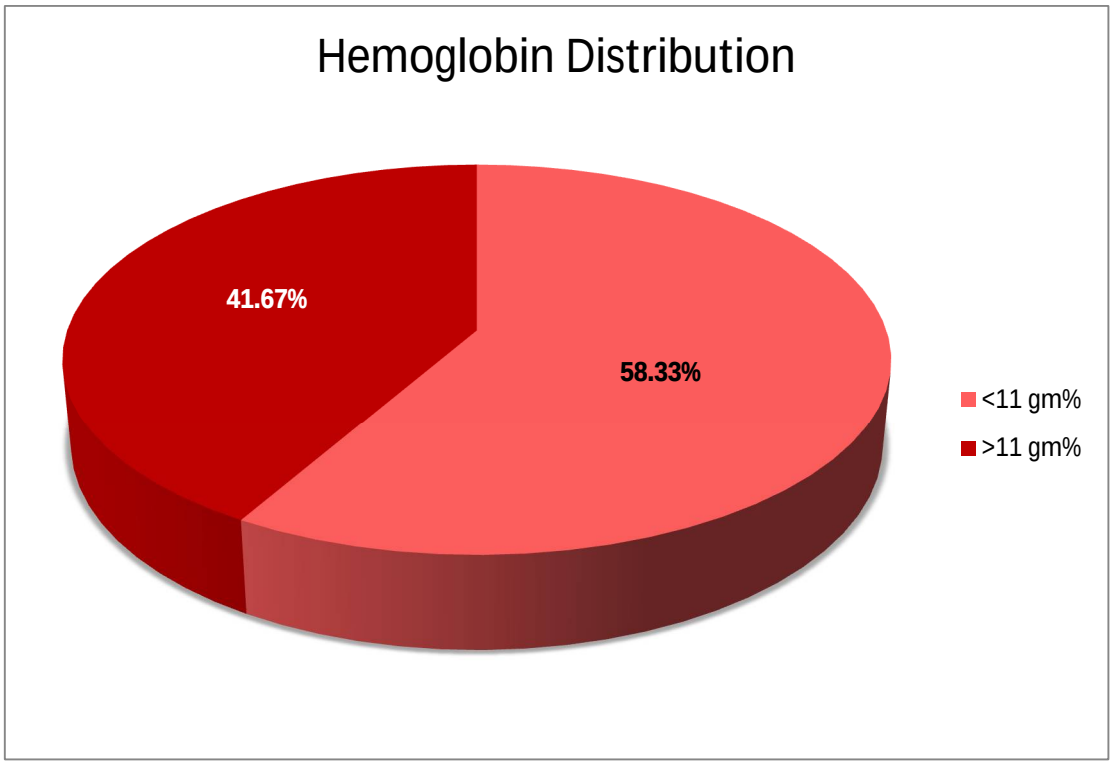
TABLE 5:

HEMOGLOBIN LEVEL:

HB	N	%
<11 GM%	35	58.33
≥11 GM%	25	41.67

HB	SALINE	PRF
<11 GM%	17	20
≥11 GM%	13	10

41.67% (25 patients) has hemoglobin more than or equal to 11 gm%. 58.3% (35 patients) has hemoglobin less than 11 gm%



WEEKLY REDUCTION IN ULCER AREA:

The weekly reduction in ulcer area was compared between control and test groups.

TABLE 6:

ULCER SIZE	CONTROL		TEST		P VALUE COMPARING CONSEQUENT WEEKS	P VALUE COMPARING CONTROL & TEST
	MEAN (cm^2)	STD. DEV.	MEAN (cm^2)	STD. DEV.		
WEEK 1	9.03	5.65	8.73	6.30	<0.01	>0.05
WEEK 2	7.91	4.61	6.13	4.56	<0.01	>0.05
WEEK 3	6.26	4.63	4.22	3.57	<0.01	>0.05
WEEK 4	4.74	3.65	2.96	2.65	<0.01	<0.05

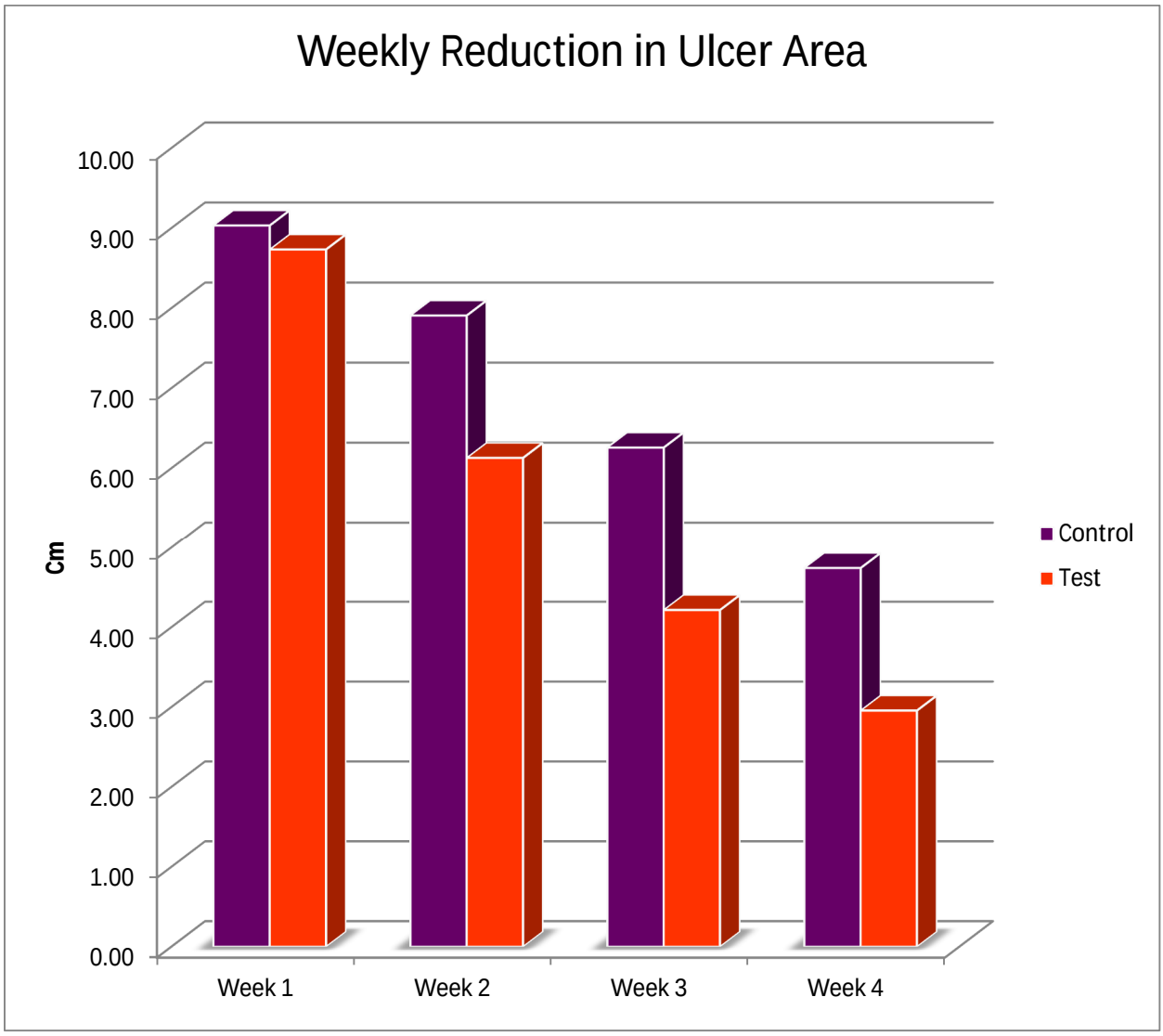

Weekly Reduction in Ulcer Area
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Cm
Week 1
Week 2
Week 3
Week 4
Control
Test

Table 7:

Percentage reduction in ulcer area at the end of four weeks:

	Control		Test		
% reduction of ulcer area at the end of 4 weeks	Mean	Standard deviation	Mean	Standard deviation	P Value
	50.5	17.4	66.9	18.2	<0.01

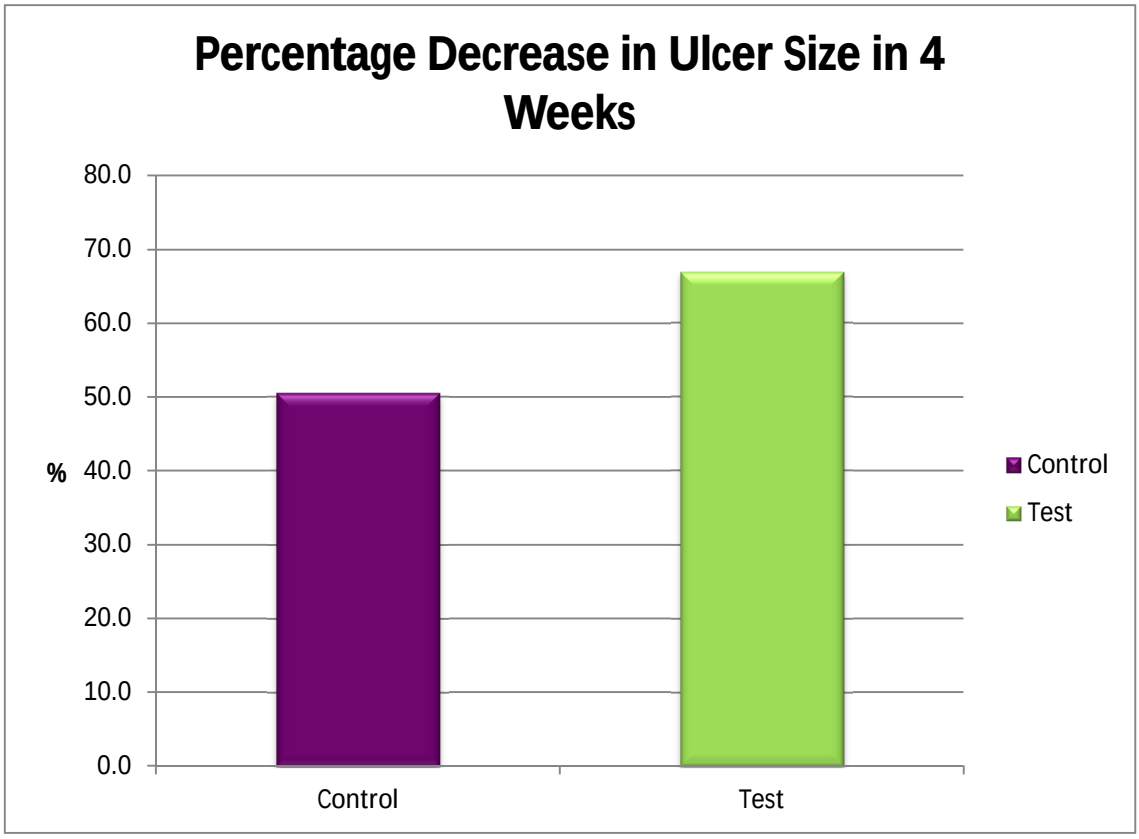


Table 8:

Molecular study:

Genotype expression in TLR 911

TLR(911)	N	Percent
AA	2	7.69
GA	11	42.31
GG	13	50
Total	26	100

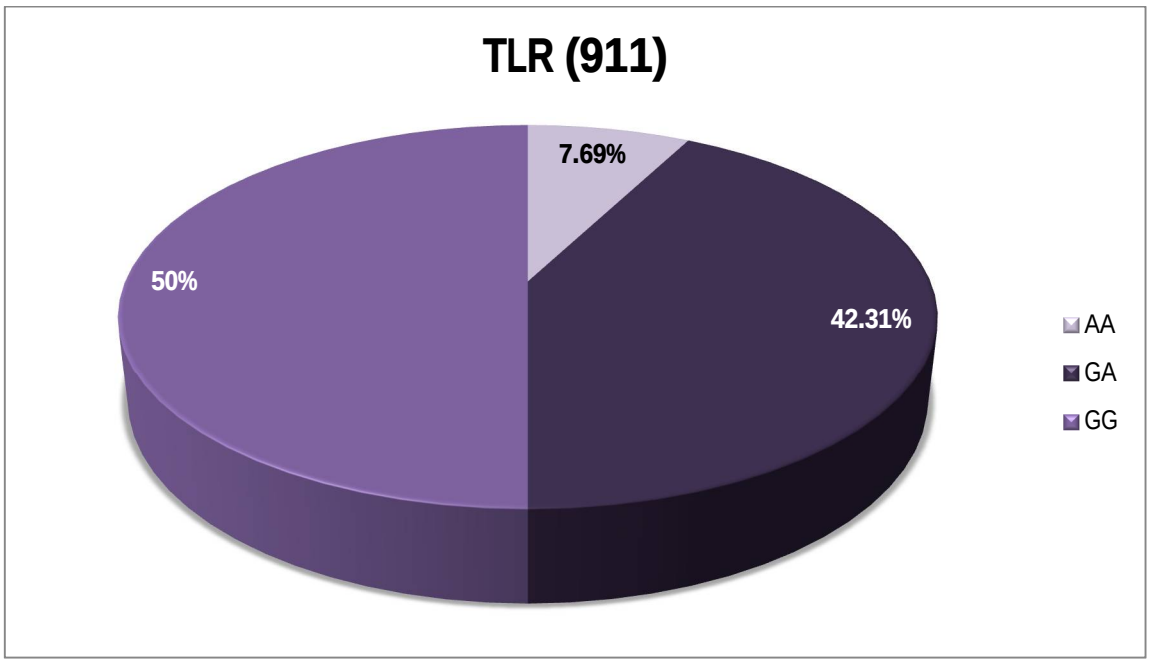


Table 9:

Genotype expression in TLR 914

TLR914	N	Percent
AA	9	34.62
GA	11	42.31
GG	6	23.08
Total	26	100

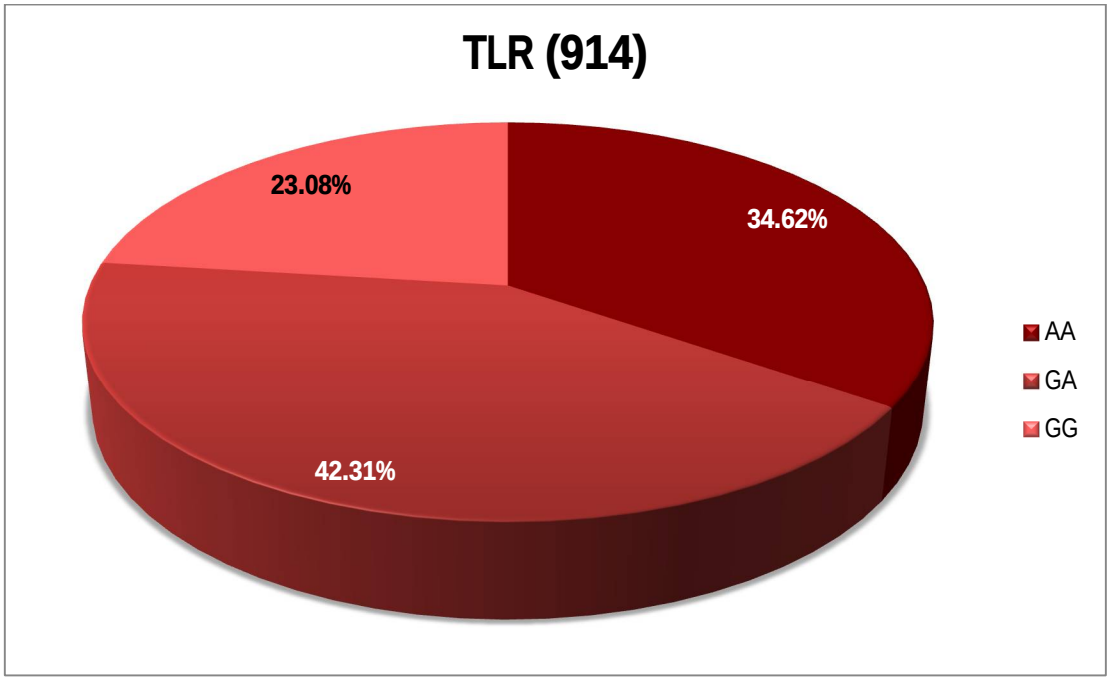


Table 10:

Gender distribution

Gender	N	Percent
F	6	23.08
M	20	76.92
Total	26	100

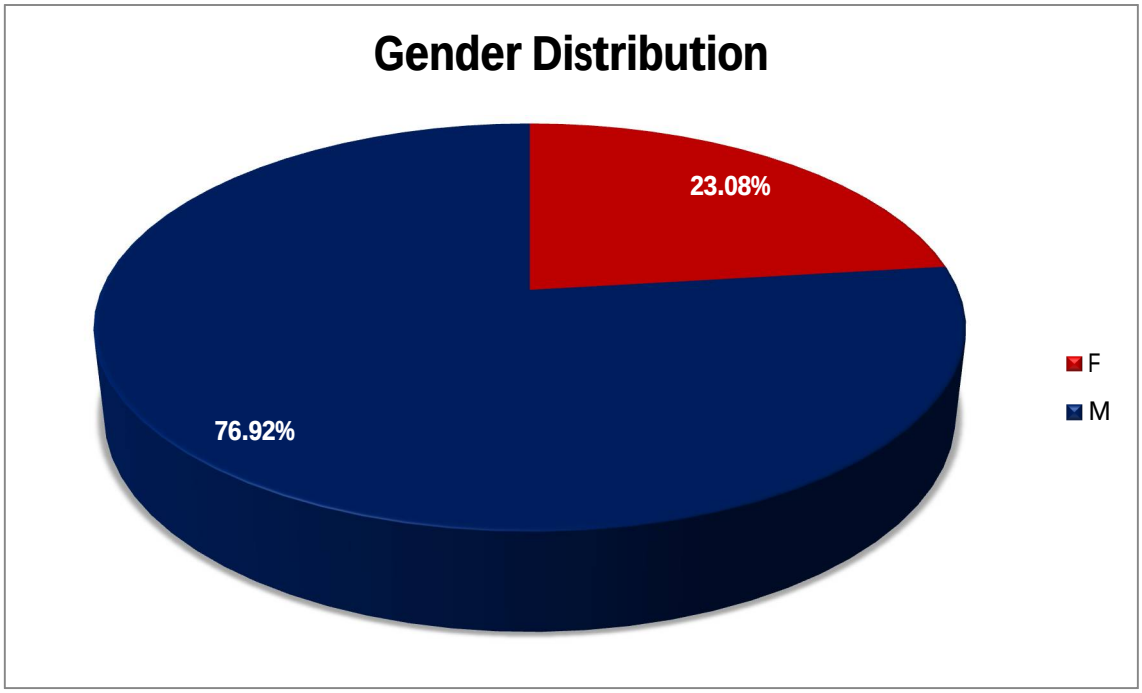


Table 11:

Percentage reduction in ulcer area at the end of 4 weeks in TLR 911:

TLR(911)	N	Mean % Reduction	SD
AA	2	44.44	15.71
GA	11	60.23	9.36
GG	13	47.92	27.14

P Value >0.05

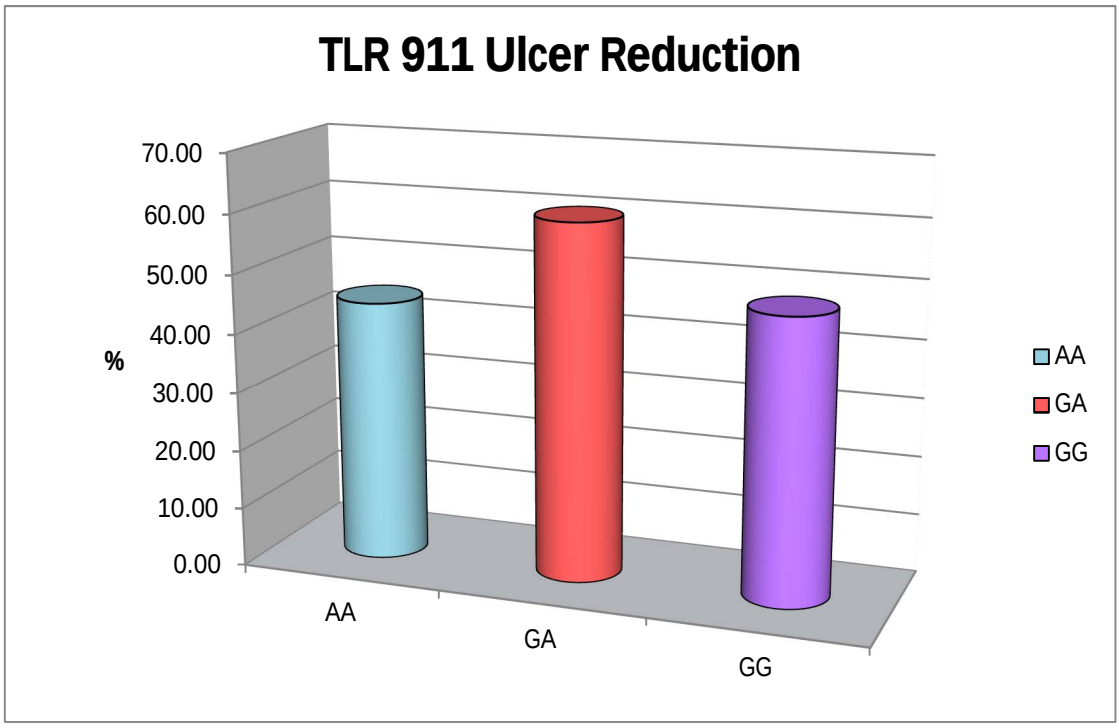


Table 12:

Percentage reduction in ulcer area at the end of 4 weeks in TLR 914:

TLR(914)	N	Mean % Reduction	SD
AA	9	55.32	20.39
GA	11	53.79	19.92
GG	6	47.45	26.39

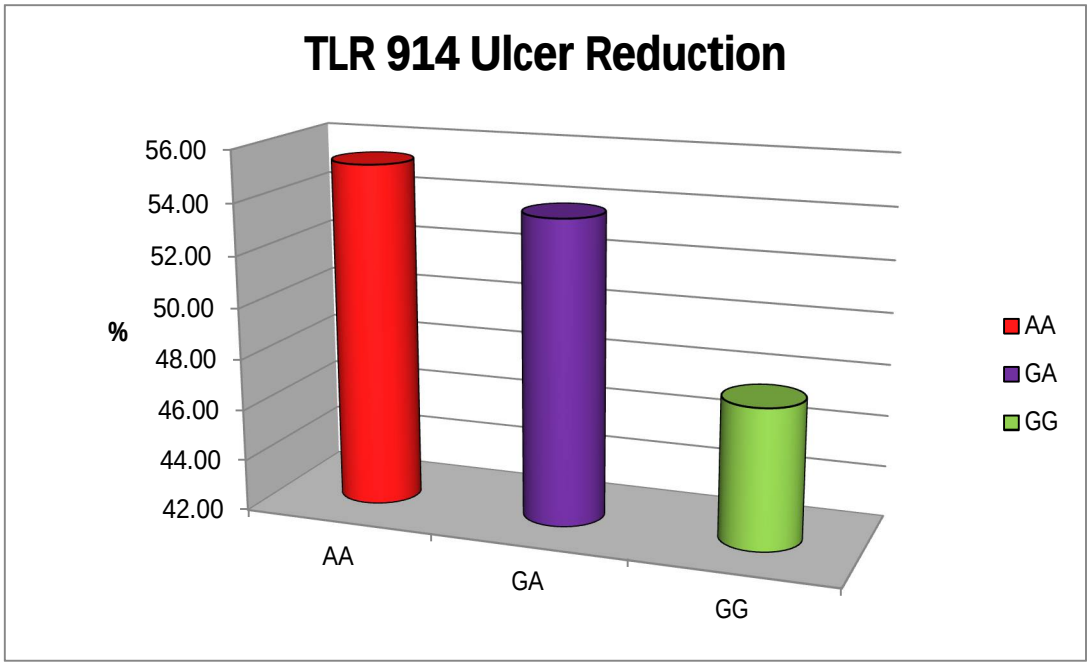


Table 13:

Comparison between PRF and Saline groups in TLR 911

	PRF			SALINE			P Value
TLR(911)	N	Mean	Standard deviation	N	Mean	Standard deviation	
AA	0			2	44.44	15.71	-
GA	5	65.33	3.15	6	55.97	10.93	>0.05
GG	8	50.52	28.99	5	43.75	26.52	>0.05

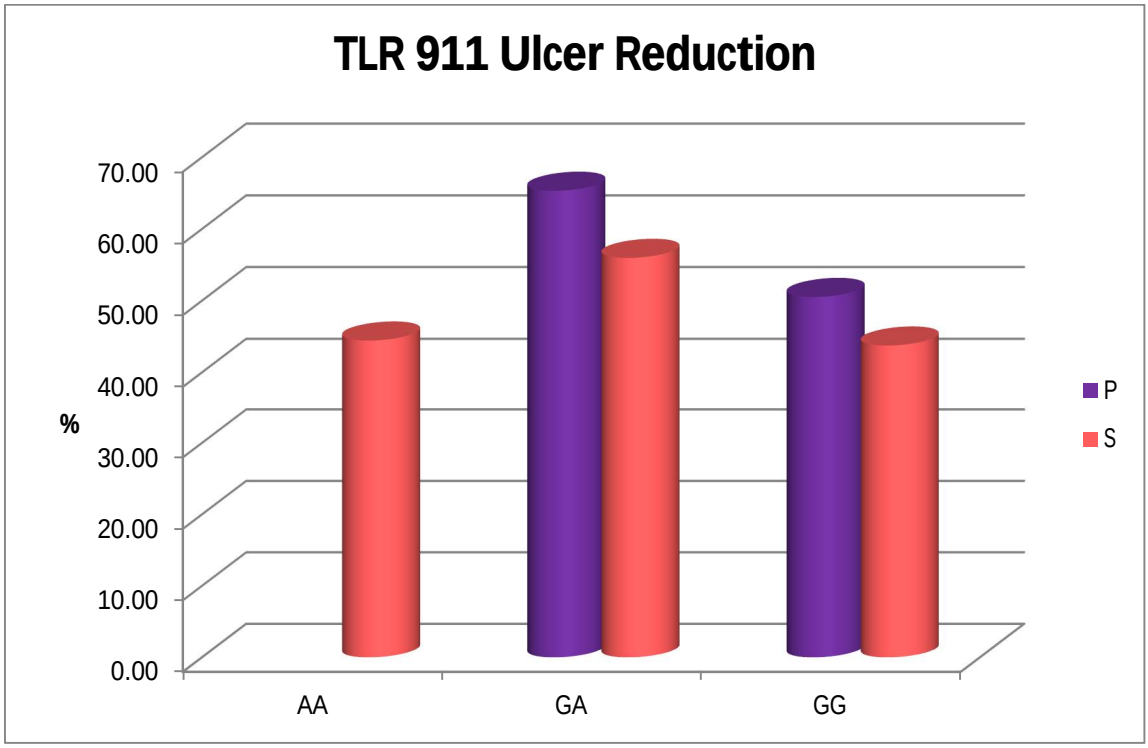


Table 14:

Comparison between PRF and Saline groups in TLR 914

	PRF			SALINE			P Value
TLR(914)	N	Mean	Standard deviation	N	Mean	Standard deviation	
AA	6	56.94	24.25	3	52.08	13.01	>0.05
GA	5	64.50	3.26	6	44.86	23.98	>0.05
GG	2	33.33	47.14	4	54.51	14.85	>0.05

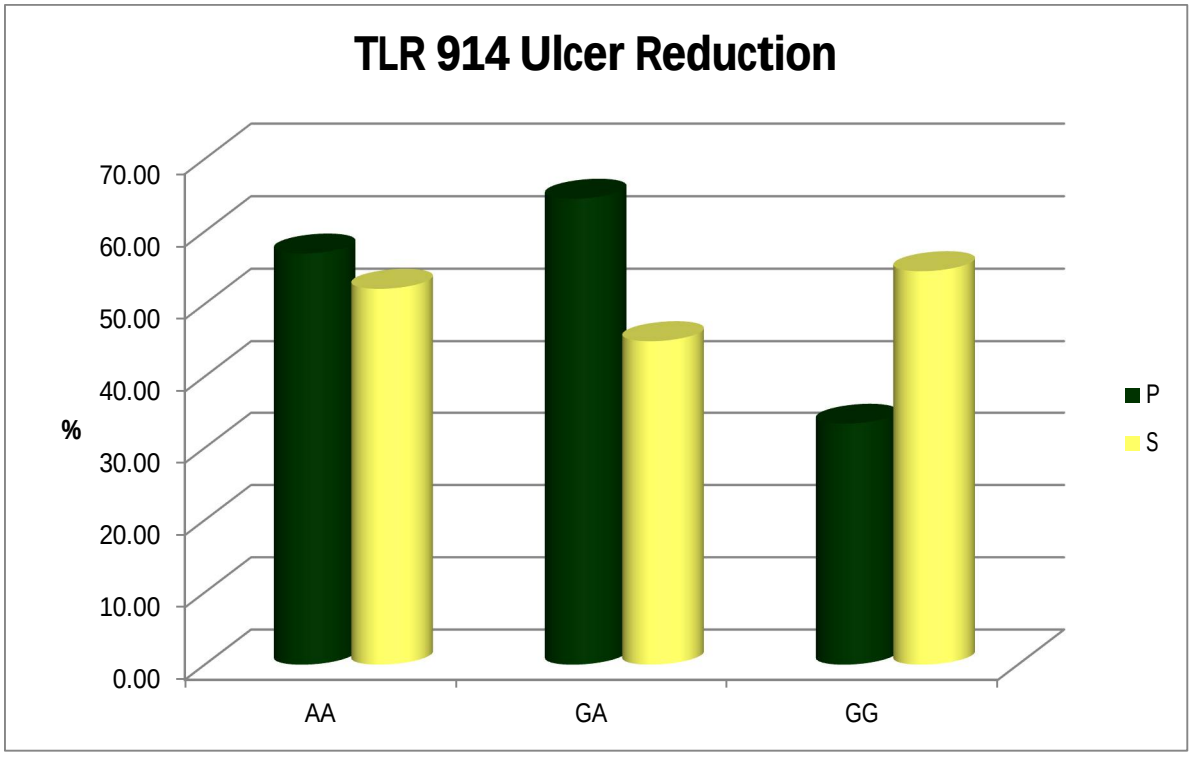


Table 17:

Evaluation of combination genotypes:

TLR911	N	Mean	SD	P Value
AA+AG	13	57.8	11.4	>0.05
GG+AG	24	53.6	12.5	

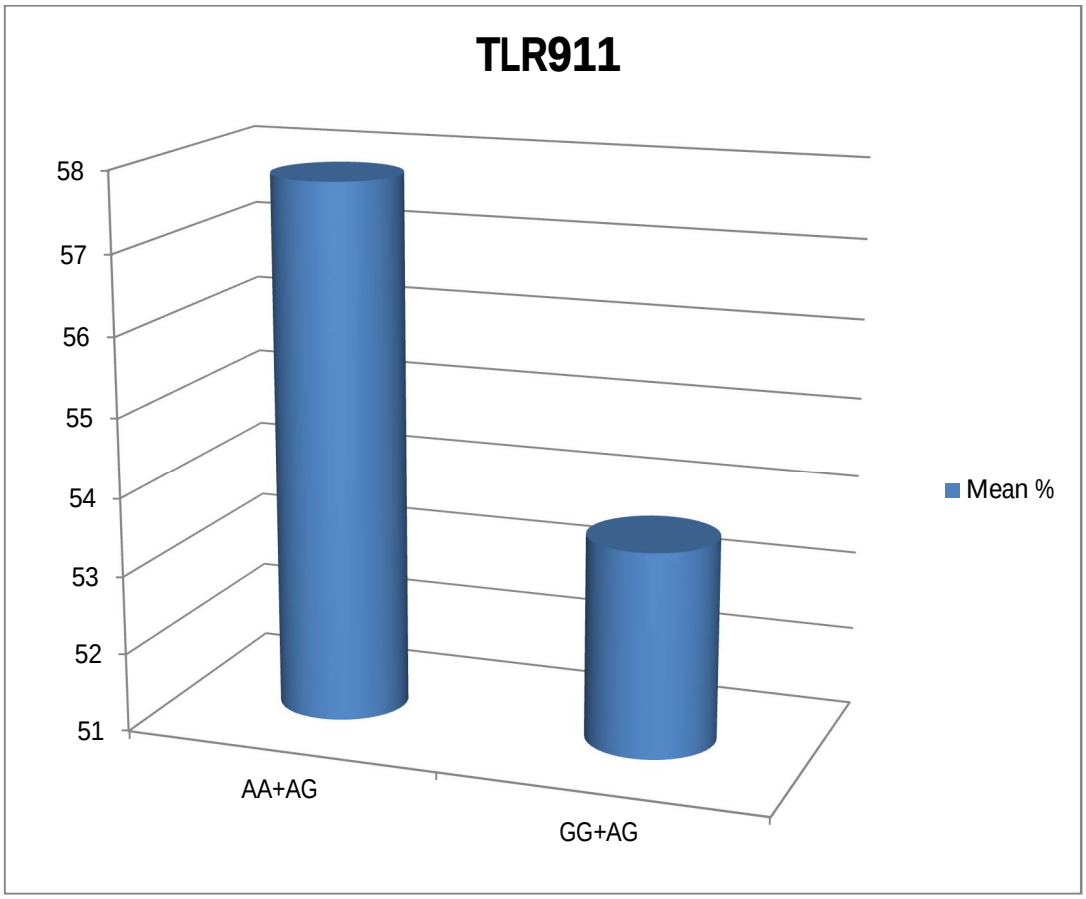
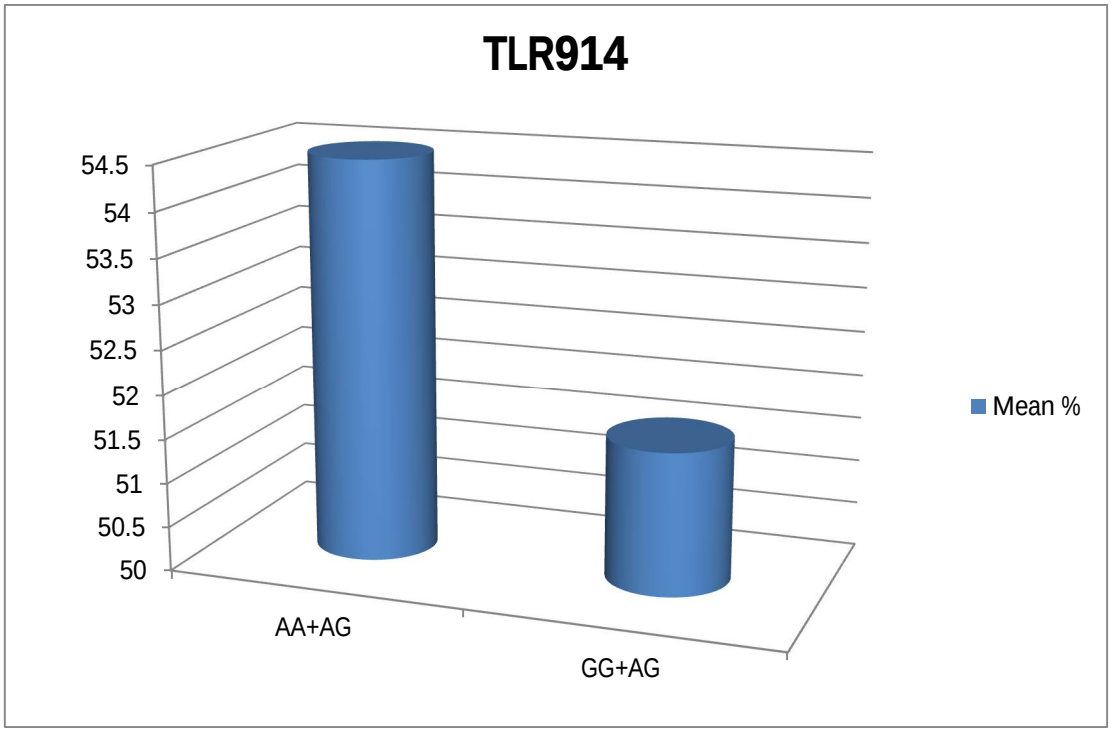


Table 18:

Evaluation of combination genotypes in TLR 914:

TLR914	N	Mean	SD	P Value
AA+AG	20	54.5	19.6	>0.05
GG+AG	17	51.6	21.8	



DISCUSSION

A randomized controlled study was done to compare the efficacy of platelet rich fibrin dressing with saline / povidone iodine dressing over a period of 4 weeks. This study also includes the analysis of the expression of TLR 4 single nucleotide polymorphisms 911 and 914 in diabetic patients with foot ulcers and their influence on wound healing.

It has been well established that diabetic foot ulcers increase the morbidity and, consequently, the financial toll on the society. Therefore, early prevention of diabetic foot ulcers is of paramount importance. Once an ulcer has formed in a diabetic patient, proper care should be instituted at the earliest to prevent it from progressing to produce extensive damage and possible need for an amputation.

Diabetic foot ulcer care involves the choice of an ideal dressing to aid in satisfactory healing of wounds. Of the many dressing materials available, platelet rich fibrin, a platelet aggregate prepared from the patient's own blood, has been the subject of research and the use of this as a dressing has been extensively studied in various fields of medicine and dentistry.

Literature review on the use of autologous platelet rich fibrin (PRF), as a dressing on diabetic foot ulcers revealed that there are not too many studies.

A study was done during 2006 on venous ulcers and PRF dressings were found to have promising results.

Valbonesi et al conducted a study of its use on non healing ulcers, wherein the results were found to favourable in 11 out of 14 non healing ulcers that were treated with platelet rich fibrin dressings.

Yazawa et al found that the growth factors concentration in platelet rich fibrin is three times more than in platelet rich plasma. It was also found that growth factors had a controlled release over a period of one week when the dressing was kept intact. The rate of epithelialisation in donor sites of split skin grafts was studied and it was found that when the donor sites were treated with PRF, 50 % re-epithelialisation was observed in 5 to 8 days when compared with controls, which was only 20%.

In this study, we compared the percentage reduction of ulcer area between the PRF group and the saline/povidone iodine group at the end of 4 weeks. The two groups of 30 patients were evenly matched. Findings in our study show that there is a significant reduction in ulcer area at the end of 4 weeks in PRF treated diabetic ulcers than in the saline/povidone iodine group. The PRF group showed 66.9 % reduction whereas the control group showed a reduction of 50.5 %.

The percentage reduction at the end of 4 weeks showed a statistical significance (p value <0.01) and it was thus concluded that PRF dressings produce better healing than saline/povidone iodine dressings. It was also found that the use of PRF dressings in diabetic ulcers can minimize the exhaustion of dressing materials. The ease of preparation of PRF makes it convenient for dressings.

There is also an economic advantage with PRF. The expense for same is much lower than that of saline/povidone iodine daily dressings. The PRF dressing can be changed once in a week which minimizes the hospital visits for the patient. Its application is thus very useful in bedridden patients, since they can be provided better wound care and wound soakage can be avoided.

The second part of this study includes the influence of Toll like receptors in wound healing. The toll like receptor, TLR 4, plays a significant role in organizing the immune pathways for tissue healing and regeneration.

A study was conducted in Varanasi in 2013 which analyzed the association of TLR 4 single nucleotide polymorphisms rs4986790, rs4986791, rs11536858, rs1927911, and rs1927914 with a high risk of diabetic foot ulcers in patients having type 2 diabetes mellitus. These SNPs are associated with impaired wound healing in diabetic patients.

On the strength of the findings of these studies, we conducted our study comparing the efficacy of PRF over saline dressings and also the expression and association of TLR 4 polymorphisms 911 and 914 with wound healing.

In this study two single nucleotide polymorphisms (SNP) of the TLR 4 receptors , TLR 911 and TLR 914 were observed in 13 patients each in PRF and the control groups. The expression of three genotypes in each namely AA, GG and GA were observed. The expressions of SNPs were compared with that of wound healing in the PRF and control groups.

It was observed that in TLR 911, the expression of GG genotype was 50%, which was more than GA which was 42.3% and AA which was 7.69%. Similarly in TLR 914 there was increased expression of GA, which was 42.31% than GA (42.31%) and AA (34.62%). So the expression of GA was found to be predominant.

When the percentage reduction in ulcer area was compared among the different genotypes in TLR 911, GA was found to have better reduction on ulcer area 60.23%, AA showed 44.44% reduction and GG showed 47.92% reduction. In TLR 914, AA showed better reduction with 55.32%, GA showed 53.79% and GG had 47.45 reduction.

We compared the test and control groups in each, between the three genotypes. In the PRF and the control groups it was observed that the GA

expression had better reduction in ulcer area with 65.33% in TLR 911 PRF treated patients and 55.97% in control. Similarly in TLR 914 it was observed that in PRF group expression of GA was associated with better reduction of ulcer area, that is, 64.50% and AA was 56.94% and GG was 33.33%.

Combination of genotype expressions were also analyzed to find the dominant allele. The combined AA + GA genotypes' analysis showed betted reduction in ulcer area in TLR 911 and in TLR 914. The expression of A was found to influence healing. However the statistical significance could not be derived. The limitation of this study was that it was conducted in a small sample size.

CONCLUSION

- Diabetic foot ulcers pose a real threat to diabetic patients in terms of morbidity and expense.
- Many dressing materials and techniques are available these days and the need for same should be tailored according to each patient.
- Platelet Rich Fibrin (PRF) when used for dressing in diabetic foot ulcers had better and faster healing when compared to saline/povidone iodine dressing.
- PRF is easy to prepare. Being an autologous preparation, it has the least adverse reactions.
- PRF has its best effect when left undisturbed for a week since it has the property of slow release of growth factors.
- Platelet Rich Fibrin dressing was found to be best suited for bed ridden patients.
- Toll like receptors 4 polymorphisms were found to influence wound healing in diabetic patients.
- It was found that the presence of allele ‘A’ in both TLR 911 and TLR 914 polymorphisms had better healing in diabetic foot ulcers treated with either saline/povidone iodine dressings or PRF dressings.

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S.NO.	IP/OP NO.	AGE	SEX	DOA/DOD	CO MORBIDS	DIABETES HISTORY	ULCER ONSET	DURATION	WOUND DETAILS	CULTURE	HB	PLATELETS	WEEK1 (LXBXW)	AREA WEEK 1 (LXB)	WEEK 2	AREA WEEK 2	WEEK 3	AREA WEEK 3	WEEK 4	AREA WEEK 4
1	I150 24154	48	M	20.8.15/29.8.15	1	NEWLY DIAGNOSED	1	4 DAYS	RIGHT LATERAL MALLEOLAR	0	10	335	5X 4 X 1	20	4X3X0.5	12	4X2.4X 0	8	3X2X0	6
2	I15027009	50	F	18.9.15/22.9.15	0	NEWLY DIAGNOSED	0	2 WEEKS	LEFT GREAT TOE CALLOUS	1	9.7	205	2X1X1	3	2X1X0.5	2	2X1X0	2	2X1X0	2
3	I15029843	54	M	14.10.15/28.10.15	0	15 YEARS	1	3 WEEKS	S/P RY AMP OF RIGHT GREAT AND 2ND TOE	0	10	180	6X4X2	24	5X3X2	15	4X3X1	12	4X2X0	8
4	I15029856	54	M	15.10.15/31.10.15	1	2 YEARS	1	1 WEEK	RAY AMP RIGHT 3RD TOE	1	9.1	471	4X3X2	12	4X2X1	8	3X1X0	3	2.5X1X0	2.5
5	I15031208	55	F	29.10.15/22.11.15	1	10 YEARS	1	1 WEEK	RAY AMP LEFT 4TH TOE	1	10.2	312	2X2X2	4	1.5X1X0.5	1.5	1X1X0	1	1X1X0	1
6	I15031749	71	M	3.11.15/	1	20 YEARS	1	6 MONTHS	2ND AND 3RD TOY AMP	1	11.1	430	6X4X2	24	5.5X3.5X1	19.25	4X3X0	12	4X2.5X0	9
7	I15030155	55	M	19.10.15/09.11.15	1	10 YEARS	1	3 MONTHS	NON HEALING ULCER LEFT HEEL	1	5.9	442	4X3X2	12	3X2X1	6	3X1.5X0	4.5	2.5X1X0	2.5
8	I15031555	41	M	1.11.15/7.11.15	1	10 YEARS	1	5 DAYS	RIGHT LATERAL MALLEOLAR	1	12	256	4X3X1	12	4X2X0	8	3.5X2X0	7	3X2X0	6
9	I15035315	50	F	2.12.15/4.12.15	1	7 MONTHS	0	2 MONTHS	DORSAL ASPECT RIGHT FOOT	1	9.9	391	4X3X1	12	4X3X1	12	4X3X0.5	12	3X3X0	9
10	I15034854	75	M	28.11.15/5.12.15	1	30 YEARS	1	10 DAYS	RAY AMP RIGHT LITTLE TOE	1	8.9	383	2X1X1	2	2X1X0.5	2	1X1X0	1	1X1X0	1
11	O15056432	63	M	OPD	0	2 YEARS	1	1 WEEK	LEFT LATERAAL MALLOLAR	0	10.2	256	3X2X1	6	INFECTED	NIL	NIL	NIL	NIL	NIL
12	I16006286	43	M	21.2.16/25.2.16	0	2 YEARS	1	1 WEEK	LEFT SOLE AND 4TH INTERDIGITAL SPACE	1	13.7	214	4X3X2	12	3.5X2.5X1	8.75	3X2X0	6	3X1.5X0	4.5
13	I16005256	55	M	13.2.16/25.2.16	1	8 YEARS	1	10 DAYS	AMP LEFT 3RD AND 4TH TOE	0	12.1	271	5X4X2	20	4.5X3X1	13.5	4X2.5X0	10	3.5X2X0	7
14	I16006244	65	M	20.2.16/26.2.16	1	8 YEARS	1	3 WEEKS	DORSUM LEFT GREAT TOE	1	12.2	423	2X2X1	4	2X1.5X0	3	2X1X0	2	1.5X1X0	1.5
15	I16012523	61	M	22.3.16/28.3.16	1	5 YEARS	0	2 WEEKS	DORSUM OF LEFT FOOT	0	12	350	4X1X1	4	4X1X1	4	3X1X0.5	3	2X0.5X0.5	1
16	I16021395	68	F	5.7.16/7.7.16	1	13 YEARS	1	1 MONTH	RIGHT SECOND TOE BASE	0	11.7	284	3X2X1	6	2.5X2X0.5	5	2X2X0	4	2X1.5X0	3
17	I16020449	62	F	27.6.16/2.6.16	1	5 YEARS	1	1 WEEK	RAY AMP RIGHT LITTLE TOE	0	11.1	284	2X2X1	4	2X2X0.5	4	1.5X1.5X0	2.25	1X1X0	1
18	I16021509	57	M	6.7.16/8.7.16	1	10 YEARS	0	1 MONTH	CALLOUS ULCER LEFT GREAT TOE	0	10.2	166	2X2X1	4	2X1.5X0.5	3	2X1.5X0	3	2X1.5X0	3
19	I16021238	58	F	4.7.16/12.7.16	1	5 YEARS	0	4 DAYS	RIGHT GREAT TOE DORSUM	1	8.9	462	3X2X1	6	2.5X1.5X0.5	3.75	2.5X1X0	2.5	2X1X0	2
20	O14074240	55	M	OPD	1	5 YEARS	1	5 DAYS	ULCER IN THE LATERAL BORDER OF HEEL	1	10.4	343	2X2X1	4	2X1.5X0.5	3	1X1X0	1	0.5X0.5X0	0.25
21	O13046550	66	M	OPD	1	2 YEARS	1	4 DAYS	ULCER IN THE DORSUM OF RIGHT FOOT	0	10.2	607	3X3X1	9	2.5X2.5X0.5	6.25	2X2X0	4	1.5X1.5X0	2.25
22	O14053015	61	M	OPD	1	5 YEARS	1	10 DAYS	S/P AMPUTATION OF RIGT GREAT TOE	1	12	232	4X3X1	12	3.5X2.5X0.5	8.75	3X2X0	6	2.5X1.5X0	3.75
23	I16025603	46	F	15.8.16/29.8.16	1	2 YEARS	1	2 MONTHS	S/P RAY AMP OF LEFT GREAT TOE	1	10	321	3X2X1	6	2.5X1.5X0.5	3.75	2X1X0	2	2X0.5X0	1
24	I16026520	59	F	23.8.16/27.8.16	1	2 YEARS	1	1 MONTH	S/P RIGHT GREAT TOE RAY AMP	1	9.6	327	3X2X1	6	2.5X1.5X0.5	3.75	2X1X0	2	2X0.5X0	1
25	I16026340	80	M	22.8.6/27.8.16	1	5 YEARS	0	1 MONTH	ULCER DORSUM OF RIGHT FOOT	0	11.1	416	4X3X2	12	3.5X2.5X1.5	8.75	3X2X1	6	2.5X1.5X0	3.75
26	I16023087	63	M	21.7.16/23.7.16	1	20 YEARS	1	1 MONTH	S/P RIGHT GREAT TOE RAY AMP	0	9.6	524	3X2X1	6	2.5X1.5X0.5	3.75	2X1X0	2	1.5X0.5X0	0.75
27	O16045172	50	F	OPD	0	2 YEARS	0	2 WEEKS	RIGHT SOLE BELOW BASE OF LITTLE TOE	0	10.5	312	2X2X1	4	1.5X1X0.5	1.5	1X0.5X0	0.5	1X0X0	1
28	O97948941	52	M	OPD	1	5 YEARS	1	1 WEEK	DORSUM OF LEFT FOOT	0	10.2	225	3X2X1	6	2.5X1.5X0.5	3.75	2X1X0	2	1.5X0.5X0	0.75
29	I16020999	65	M	1.7.16/7.7.16	0	3 YEARS	1	15 DAYS	S/P I AND D OF LEFT LEG ABSCESS	0	12.3	254	2X1X1	2	1.5X1X0.5	1.5	1X0.5X0	0.5	1X0X0	1
30	O15039584	61	F	OPD	1	2 YEARS	0	3 WEEKS	SOLE OF LEFT FOOT NEAR BASE OF 4TH TOE	0	13.7	345	2X2X1	4	1.5X1.5X0.5	2.25	1X1X0	1	0.5X0.5X0	0.25

S.NO	IP NO	AGE	GENDER	CO MORBIDS	DIABETIC STATUS	ULCER ONSET	DURATION	ULCER DETAILS	WOUND CULTURE	HB	PLATELETS	1ST WEEK	AREA WEEK 1	2ND WEEK	AREA WEEK 2	3RD WEEK	AREA WEEK 3	4TH WEEK	AREA WEEK 4
1	I15024914	52	F	1	3 YEARS	0	15 DAYS	MEDIAL ASPECT LEFT FOREFOOT	1	10.2	288	4X3X1	12	4X3X1	12	3X2X1	6	3X2X1	6
2	I15031342	51	F	0	2 YEARS	1	1 WEEK	DORSUM OF RIGHT FOREFOOT	0	11	238	3X3X1	9	3X3X1	9	2.5X2.5X1	6.25	2X2X.5	4
3	I16031208	62	F	1	5 YEARS	0	3 DAYS	4TH TOE RAY AMP	1	10.2	277	2X2X1	4	2X1.5X1	3	2X1X1	2	1.5X1X0.5	1.5
4	I15068311	57	M	1	10 YEARS	0	1 MONTH	MULTIPLE ABSCESS SITE ULCER RIGHT SOLE	0	8	210	5X4X1	20	5X3.5X1	17.5	4.5X3.5X1	15.75	4X3X0.5	12
5	I15033287	62	F	1	15 YEARS	0	3 DAYS	PLANTAR ASPECT LEFT FOREFOOT	0	10.9	458	2X2X1	4	2X1.5X1	3	2X1X1	2	1.5X1X0.5	1.5
6	O15072735	65	M	0	5 YEARS	1	1 WEEK	DORSUM OF RIGHT FOOT	0	12.3	263	5X4X1	20	5X3.5X1	17.5	4.5X3X0.5	13.5	4X2.5X0.5	10
7	O15042073	65	F	0	10 YEARS	1	2 WEEKS	PLANTAR ASPECT OF RIGHT FOOT NEAR HEEL	1	8.2	460	4X2X1	8	4X2X1	8	4X2X1	8	4X2X1	8
8	O15077871	45	M	0	RECENTLY DIAGNOSED	1	1 WEEK	NEAR LEFT LATERAL MALLEOLUS	0	12	265	2X2X1	4	2X1.5X1	3	2X1X1	2	1.5X1X0.5	1.5
9	I15034524	62	M	1	12 YEARS	0	4 DAYS	S/P RIGHT GREAT TOE DISARTICULATION	1	11.3	271	3X2X1	6	3X2X1	6	2.5X1.5X1	3.75	2X1X0.5	2
10	I15034129	48	M	1	10YEARS	0	1 YEAR	LEFT FOOT ABSCESS	0	10.1	385	5X3X1	12	4.5X2.5X1	11.25	4.5X2.5X0.5	11.25	4X2X0.5	8
11	I15035221	54	M	1	10 YEARS	1	3 MONTHS	RIGHT 4TH TOE DORSUM	1	12.3	288	4X3X1	12	3.5X2.5X0.5	8.75	3X2X0.5	6	3X1.5X0	4.5
12	I15035170	55	M	1	5 YEARS	0	3 DAYS	MEDIAL ASPECT LEFT FOREFOOT	1	12.6	326	4X3X2	12	4X2.51.5	10	3.5X2X1	7	3.5X1.5X0.5	5.25
13	I15034952	52	M	1	6 YEARS	1	1 WEEK	RIGHT MEDIAL ASPECT OF PLANTAR ASPECT	0	10.4	415	3X2X1	6	3X1.5X1	4.5	2.5X1.5X1	3.75	2X1X0.5	2
14	I15035866	60	M	1	10 YEARS	0	1 WEEK	LATERAL MALLELUS	0	8.4	203	4X1X1	4	4X1X1	4	3.5X0.5X1	1.75	3X0.5X0.5	1.5
15	I16000020	63	F	1	10 YEARS	0	4 DAYS	S/P RIGHT GREAT TOE DISARTICULATION	0	10.3	324	3X2X1	6	3X1.5X0.5	4.5	2.5X1.5X0.5	3.75	2X1X0.5	2
16	O0045321	57	M	1	3 YEARS	0	1 DAY	RIGHT HEEL	0	13.1	256	3X2X1	6	3X2X1	6	3X1.5X1	4.5	2.5X1X0.5	2.5
17	I16001345	53	M	0	2 YEARS	1	1 WEEK	S/P LEFT LITTLE TOE DISARTICULATION	1	12.2	250	3X2X1	6	3X1.5X0.5	4.5	2.5X1.5X0.5	3.75	2X1X0.5	2
18	I16023454	60	M	1	6 YEARS	0	2 WEEKS	LEFT SOLE POST DEBRIDEMENT	1	10.4	203	5X4X1	20	4.5X3.5X0.5	15.75	4.5X3.5X0.5	15.75	4X3X0	12
19	I16012678	57	M	0	RECENTLY DIAGNOSED	0	1 WEEK	RIGHT FOREFOOT	0	12.1	213	4X3X1	12	3.5X3X1	10.5	3X2.5X1	7.5	2.5X2.5X1	6.25
20	I16015888	52	F	0	5 YEARS	1	2 WEEKS	S/P WOUND DEBRIDEMENT - RIGHT FOREFOOT	0	7.9	241	3X2X1	6	3X2X1	6	2.5X1.5X1	3.75	2.5X1.5X1	3.75
21	I16016561	60	F	0	2 YEARS	1	1 WEEK	RIGHT HEEL	0	9.3	410	2X2X0	4	2X2X0	4	2X1.5X0	3	2X1.5X0	3
22	I16015832	46	M	0	1 YEAR	1	1 WEEK	DORSUM OF RIGHT FORE FOOT	0	12	345	3X2X1	6	3X2X1	6	2.5X2X1	5	2X1X0.5	2
23	O09033817	52	F	1	1 YEAR	1	1 WEEK	LEFT HEEL	0	11.5	422	3X2X1	6	3X2X1	6	2.5X1.5X1	3.75	2.5X1.5X0.5	3.75
24	I16025826	58	M	1	10 YEARS	0	1 MONTH	LEFTLATERAL ASPECT OF HEEL	1	7	208	5X4X1	20	4.5X3.5X1	15.75	4.5X3.5X1	15.75	4X3X0.5	12
25	O1606253	55	M	1	5 YEARS	1	3 DAYS	RIGHT MEDIAL MALLEOLUS	1	11.5	412	3X2X1	6	3X2X1	6	2.5X1.5X1	3.75	2.5X1.5X1	3.75
26	I16021283	70	M	1	10 YEARS	0	2 WEEKS	DORSUM OF LEFT FOOT	0	9.1	279	4X1X1	4	4X1X1	4	3.5X0.5X1	1.75	3X0.5X0.5	1.5
27	I16020145	65	M	1	4 YEARS	0	1 WEEK	DORSUM OF LEFT FOOT	1	8.6	341	5X4X1	20	4.5X3.5X1	15.75	4.5X3.5X1	15.75	4X3X1	12
28	O16042017	50	M	1	2 YEARS	0	2 WEEKS	LEFT ANKLE	0	12.4	227	3X2X1	6	3X2X1	6	2.5X1.5X1	3.75	2X1X0.5	2
29	I16022624	60	M	1	10 YEARS	1	1 WEEK	BASE OF LEFT THIRD TOE	0	9.8	381	2X2X1	4	2X1.5X1	3	1.5X1.5X1	2.25	1X1X0.5	1
30	I16026233	40	M	1	7 YEARS	1	2 WEEKS	DORSUM OF LEFT FOOT	0	8.7	223	3X2X1	6	3X2X1	6	2.5X2X1	5	2.5X2X1	5

s.no	sample code	Treatment	sex	op/ip number	TLR(911)	TLR914	area week 1	area week 4	% reduction
1	PRF1	P	M	I15024154	GA	GA	20	6	
2	PRF2	S	F	I15024714	GA	GA	12	6	
3	PRF3	P	F	I15027009	GG	AA	3	2	
4	PRF4	P	M	I15027009	GA	GG	24	8	
5	PRF5	P	M	I15029856	GG	AA	12	2.5	
6	PRF6	P	F	I15031208	GG	AA	4	1	
7	PRF7	P	M	I15031749	AG	GA	24	9	
8	PRF8	P	M	I15030155	GG	AA	12	2.5	
9	PRF10	S	F	I15033422	AA	GG	9	4	
10	PRF11	S	F	I15031208	GG	AA	4	2.5	
11	PRF12	S	M	I1503102	AG	GA	20	12	
12	PRF13	S	F	I1503287	GG	AA	4	1.5	
13	PRF14	S	M	O15072735	AG	GA	20	10	
14	PRF15	S	M	O15042073	GG	GA	8	8	
15	PRF-16	S	M	O15077871	GG	GA	4	1.5	
16	PRF17	S	M	I15034524	AG	GA	6	2	
17	PRF18	S	M	I15034129	AA	GG	12	8	
18	PRF19	P	M	I15035315	GG	AA	12	9	
19	PRF20	P	M	I15034854	GG	AA	2	1	
20	PRF21	S	M	I15035221	AG	GG	12	4.5	
21	PRF22	S	M	I15035170	GG	AA	12	5.25	
22	PRF23	S	M	I15034952	AG	GG	6	2	
23	PRF24	P	M	O150	GG	GG	6	nil	
24	PRF26	P	M	I16006286	GA	GA	12	4.5	
25	PRF27	P	M	I16005256	AG	GA	20	7	
26	PRF28	P	M	I1606244	GG	GA	4	1.5	

ANNEXURE

PRF	-	Platelet Rich Fibrin
PRP	-	Platelet Rich Plasma
TLR	-	Toll Like Receptor
SNP	-	Single Nucleotide Polymorphism
HB	-	Hemoglobin
RFLP	-	Restriction Fragment Length Polymorphism
PCR	-	Polymerase Chain Reaction